Legionella in habitations

Detection and risk factors

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DTU Environment
Department of Environmental Engineering

PhD Thesis
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Preface

The ideas behind this PhD thesis have emerged in collaborations between the Department of Environmental Engineering at the Technical University of Denmark and the Department of Microbiological Surveillance and Research, Statens Serum Institut. The timeframe for the project was from April 2008 to May 2011. The funding was 1/3 from DTU, 1/3 from the Urban water Technology Graduate School and 1/3 from Statens Serum Institut. Three supervisors are affiliated with the project: Professor Hans-Jørgen Albrechtsen, Senior Researcher Søren Anker Uldum and Professor Karen Angelika Krogfelt. All laboratory work was conducted at Statens Serum Institut.

The following papers compose the basis of the thesis and have either been published or submitted.

I. Krøjgaard L.H., Krogfelt K. A., Albrechtsen H-J. Uldum S.A. Cluster of Legionnaires´ disease in a newly built block of flats, Denmark, December 2008-January 2009 Euro Surveill. 2011; vol. 16, issue 1, 06 jan 2011.

II. Krøjgaard L.H., Krogfelt K. A., Albrechtsen H-J. Uldum S.A Validation of a quantitative real time PCR (qPCR) for detection of *Legionella* spp and *Legionella pneumophila* in water samples.
Submitted

III. Krøjgaard L.H., Krogfelt K. A., Albrechtsen H-J. Uldum S.A Detection of *Legionella* by quantitative-polymerase Chain reaction (qPCR) for monitoring and risk assessment.
Submitted

The first article will be referred to as Krøjgaard *et al.* 2011, and the two other articles will be referred to with the Roman numerals accentuated in bold (e.g., Krøjgaard *et al.* **II** and Krøjgaard *et al.* **III**). The same water samples are the root of both Krøjgaard *et al.* 2011 and Krøjgaard *et al* **III**.

The same samples are used in article **I** and **III**. In article **I** results are based on culture, in article **III** results are also based on qPCR (quantitative real-time polymerase chain reaction).

Accepted abstracts:

Krøjgaard L.H., Albrechtsen H-J., Krogfelt K.A., Uldum S.A. Microbial risk assessment of urban water: Development of methods for detection and analysis of pathogens with Legionella as a model organism. A q-PCR method for risk assessment and source investigation. Urban Water Technology-Graduate School-UWT, Yearly Seminar 27-28 may 2009 Brædstrup, Denmark.
Presented as a 20 min oral presentation.

Krøjgaard L.H., Krogfelt K.A., Albrechtsen H-J., Uldum S.A. Legionnaires´ disease associated with a new residential area -Risk factors and remedial actions. *Legionella* 2009 conference, 13-17 October 2009 Paris, France.
Presented as a poster.

Krøjgaard L.H., Albrechtsen H-J., Krogfelt K.A.,Uldum S.A Microbiological risk assessment of urban water. Development of methods for detection and analysis of pathogens with *Legionella* as model organism. Preparing DNA standards for Quantitative Real Time PCR for quantification of *Legionella* in water samples. Urban Water Technology - Graduate School - UWT, Yearly Seminar 31 May-1 June 2010, Copenhagen Denmark.
Presented as a 20 min oral presentation.

Krøjgaard L.H., Krogfelt K.A., Albrechtsen H-J., Uldum S.A. Risk assessment and monitoring of *Legionella* by culture and qPCR in a newly build block of flats associated with a small outbreak of Legionnaires´ disease *Legionella* 2010 conference 15-17 September 2010, Copenhagen Denmark.
Presented as a poster.

Krøjgaard L.H., Krogfelt K.A Albrechtsen H-J., Uldum S.A Two cases of Legionnaires´ disease associated with a newly build residential area - risk factors and remedial actions. Forskningsplatformen –vand conference 28-29 January 2011, Copenhagen, Denmark.
Presented as a 20 min oral presentation.

The papers are referred to as roman numerals **I-III**. They are not included in this www-version but can be obtained from the library at DTU Environment, library@env.dtu.dk or Department of Environmental Engineering, Technical University of Denmark, Miljoevej, building 113, DK-2800 Kgs. Lyngby, Denmark

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A special thank you goes to my office room mate at Statens Serum Institute**,** Department of Microbiologic Diagnostic**,** Lillian Marie Søes MD PhD, for always making ours a nice working environment in the 'green' office. Thanks also go technical designer Torben Dolin, The Technical University of Denmark, Department of Environmental Engineering, for help with some of the graphic challenges in the presentation process.

Finally, I wish to thank my family and friends for support and fun throughout a sometimes stressful period.

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Louise Hjelmar Krøjgaard

Abstract

Legionella is the causative agent of Legionnaires' disease. The bacteria are widespread in nature and man-made water systems. In Denmark, approximately 120 cases are diagnosed each year, and the disease can be fatal. *Legionella pneumophila* is the species responsible for approximately 95% of cases. The transmission pathway is through inhalation of contaminated water droplets mainly from technical systems such as hot water systems.

Overall, the aim of this PhD thesis was to improve the background knowledge to accomplish risk assessment regarding *Legionella* in water systems. Based on a literature review and the results from the PhD work the following subjects were addressed: a) prevalence of *Legionella* in habitations, b) validation of the use of qPCR in risk assessment in hot water systems, c) clarifying risk factors mainly associated with *Legionella* in habitations, and d) discussion of interventions which could be used to overcome or prevent a *Legionella* colonisation in water.

The standard method to quantify *Legionella* in water samples is culturing, but since it has long response time (7-14 days) faster methods are needed to evaluate if *Legionella* is present and to quantify the numbers to assess the risk. Quantitative real-time polymerase chain reaction (qPCR) is an alternative and /or a supplement and two qPCR assays targeting *Legionella* species and *Legionella pneumophila* were implemented and validated. Limit of detection for *Legionella* species was found to be 833 GU/L and for *Legionella pneumophila* 5000 GU/L. Limits of quantification of the assays were 3333 GU/L for the *Legionella* species assay, and 8333 GU/L for the *Legionella pneumophila* assay. The efficiency was 91.6% and 96.6% respectively.

Both assays were tested on real life water samples from mixed sources (cooling towers, hospital water, schools and private residents). Analysing these randomly collected samples with the qPCR assay targeting *Legionella pneumophila* and traditional culture good correlation (N = 43, r=0.77) was found.

The assays were also applied in a risk assessment of a newly built residential area with a cluster of Legionnaires´ disease cases. These samples also included sampling before and after interventions such as thermal treatment and hyperchlorination. When all samples from this location were pooled the quantification of *Legionella* by q-PCR and by culture did not correlate well.

However, when the samples were grouped according to their type and how they were collected, such as e.g. ‘circulation water’ and ‘water from first flush from shower hoses’, culture and qPCR showed the same tendencies. Because the ranges of *Legionella* concentration found by qPCR between and after the thermal treatments overlapped, it was difficult to interpret the specific amount. In samples collected from the first flush from empty apartments, culture and qPCR were inconclusive.

The literature studies showed that *Legionella* is widely dispersed in habitations all over the world, including in Denmark. Different major risk factors were identified: Temperature not sufficient to suppress growth of *Legionella*. Water tapped from water systems using centralised heating or distant heating was more often colonised than water from systems with instantaneous heaters (no water tank). Most studies showed that copper material suppressed growth of *Legionella.* Presence of other bacteria and amoebae had a positive effect on growth and survival of *Legionella*, since *Legionella* situated inside amoebae were better protected against thermal treatments than free in the water phase.

Different treatments can be implemented to overcome *Legionella* colonisation. Other have shown that more permanent, long-term water treatments, such as copper-silver ionisation, addition of chlorine dioxide or monochloramine to the portable water can be effective against *Legionella* colonisation, though none of the methods completely eradicated *Legionella* in all treatet water systems. However, in case of an outbreak/cluster immediate interventions are needed. Two immediate treatments (thermal treatment and hyperchlorination) were investigated in a newly built residential area with a cluster of Legionnaires’ disease cases. The newly built residential area constituted the main area for this PhD study. Raising the temperature in the boilers to 70°C for 24 hours followed by three weeks at 65°C in the boilers, flushing of all taps and shower hoses and hyperchlorination of boilers caused a notable decrease in the number of *Legionella.* Before the thermal treatment, circulation water contained up to 1.2 * 10^4 colony-forming units (CFU)/L, but after the treatment, no or very limited *Legionella* was observed by culture for at least seven months. An important factor when controlling *Legionella* in a water system is the daily operation of the system. Water should be > 50°C at all taps.

Dansk resumé

Legionella er årsag til legionærsygdom. Bakterien findes både i naturen og i menneskeskabte vandsystemer. I Danmark er der hver år omkring 120 diagnosticerede tilfælde og sygdommen kan have dødelig udgang. *Legionella pneumophila* er den art der giver ophav til omkring 95 % af sygdomstilfældene Smitte sker ved indånding af forurenede vandpartikler hovedsagelig fra tekniske systemer som varmtvandssystemer.

Overordnet var formålet med denne PhD afhandling at danne baggrund for en bedre baggrundsviden at foretage risikovurdering angående *Legionella* i vandsystemer ud fra. Baseret på litteratur studier og resultater fra eget PhD arbejde blev følgende emner behandlet: a) udbredelsen af *Legionella* i beboelse, b) validering af brugen af qPCR i risikovurdering i varmtvandssystemer c) fremlægge risikofaktorer hovedsagelig i forbindelse med *Legionella* i beboelse og d) diskutere de metoder der er til at bekæmpe eller forebygge kolonisering af *Legionella* i vand.

Standard metoden til at kvantificere *Legionella* i vand er dyrkning, men da svartiden er lang (7-14 dage), er der behov for hurtigere metoder til at bestemme om *Legionella* er til stede og i hvilket antal for at vurdere risikoen. Quantitativ real-time polymerase chain reaction (qPCR) er et alternativ og/eller et sublement, og qPCR assays rettet mod *Legionella* arter og *Legionella pneumophila* blev implementeret og valideret. Detektions grænsen for *Legionella* species blev fundet til at være 833 GU/L og for *Legionella pneumophila* 5000 GU/L. Kvantifikationsgrænsen var 3333 GU/L for *Legionella* species assayet og 8333 GU/L for *Legionella pnuemophila* assayet. Effektiviteten var henholdsvis 91.6% og 96.6%.

Begge assay blev testet på naturlige prøver fra flere steder (køletårne, hospitaler, skoler og privat beboelse). Når disse tilfældigt indsamlet prøver blev analyseret med qPCR assay rettet mod *Legionella pneumophila* og dyrkning var der god koorelation (N=43, r=0.77).

Begge assay blev også anvendt i forbindelse med risikovurdering af et nybygget beboelsesområde hvor en klynge af legionærsygdomstilfælde er associeret til. Disse prøver inkluderede prøver indsamlet før og efter interventioner så som varme behandling og hyperklorering. Når alle prøver fra denne lokalitet blev

samlet og testet uden hensyn til indsamlingstid og sted, korrelerede antallet fundet ved qPCR og dyrkning ikke særlig godt. Blev prøverne derimod inddelt afhængig af deres type og hvordan de var tappet så som ” cirkulations vand ” og ”vand fra første skyl fra bruseslanger”, viste dyrkning og qPCR de samme tendenser. Pga. mængderne af *Legionella* fundet ved qPCR før og efter varmebehandlingerne overlappede, var det svært at tolke på det konkreate antal verificeret ved qPCR. I prøver indsamlet som den første liter vand fra tomme lejligheder var resultaterne fra dyrkning og qPCR ikke entydige.

Litteratur studiet viste at *Legionella* er vidt udbredt i beboelse over hele verden og også i Danmark. Forskelige vigtige risiko faktorer blev identificeret: Temperatur der ikke hæmmer vækst af *Legionella*. Vand fra vandsystemer hvor der anvendes centralvarme eller fjernvarme var hyppigere koloniseret end vand fra systemer med øjeblikkelig varme (hvor der ikke anvendes varmetank). Kobber materiale viste i de fleste undersøgelser hæmmende effekt på vækst af *Legionella*. Tilstedeværelsen af andre bakterier og amøeber havde en positiv effekt på vækst og overlevelse af *Legionella*, da *Legionella* inde i amøber var bedre beskyttet mod varmebehandlinger end når de var fri i vandfasen.

Forskellige behandlinger kan inplementeres for at overvinde en *Legionella* kolonisering. Andre har vist at mere permanente langtids vandbehandlinger så som kobber-sølv ionisering, tilsætning af klordioxid eller monokloramin til ledningsvandet kan være effektivt mod en *Legionella* kolonisering trods ingen af metoderne totalt udrydede *Legionella* i alle testede vandssystemer. I tilfælde af et *Legionella* udbrud/klynger af tilfælde kræves dog omgående interventioner. To omgående interventioner (varmebehandling og hyperklorering) blev testet i et nybygget beboelsesområde hvor en klynge af tilfælde med legionærsygdom var associeret til. Området udgør det største område for indsamling af prøver i denne PhD. Øget temperaturen i kedlerne til 70 ºC i 24 timer efterfulgt af tre uger med 65 ºC i kedlerne, skyldning af alle haner og bruse slanger, og hyperkliorering af kedler medførte et tydelig fald i antallet af *Legionella*. Før varmebehandlingen indeholdt cirkulationsvandet op til 1.2 $*10^4$ koloni-formende enheder (CFU/L), men efter behandlingen og mindst 7 måneder frem blev kun få hvis nogen *Legionella* overhovedet, observeret vha dyrkning. En vigtig faktor for at kontrollere *Legionella* i et vandsysten er den daglige drift. Vand skal ved alle tapsteder være >50 ºC.

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1 Introduction

The term Legionnaires' disease was coined after a severe outbreak in July 1976 at a Belleveue-Stratford hotel hosting the 58th annual convention of the American Legion in Philadelphia, Pennsylvania. Two hundred twenty-one convention attendees were infected, and 34 died (Rosa 1993). The causative agent of the outbreak was found to belong to a family of bacteria later named Legionellaceae. Today *Legionella*ceae has at least 50 species and 70 distinct serogroups. Not all species are pathogenic, and *Legionella pneumophila* is the causative agent of more than 95% of all *Legionella* infections in Europe (Joseph & Ricketts 2010, Bartram *et al.* 2007).

The symptoms of infection are diverse, from mild febrile illness called Pontiac fever to rapid and potential fatal pneumonia (Legionnaires' disease) (Bartram *et al.* 2007). Not everyone exposed to the bacteria develops symptoms. The attack rate for Pontiac fever is much higher than for the development of Legionnaires' disease (Bartram *et al.* 2007).

Legionella is widely dispersed in natural water sources and in humid environments (Cramp *et al.* 2010, Rosa 1993, Fliermans *et al.* 1981). It is also found in high numbers (>10 ^{4} CFU/L) in man-made water systems like cooling towers (Ferre *et al.* 2009, Castilla *et al.* 2008), spas (Guillemet *et al.* 2010) and warm water systems (Krøjaard *et al.* 2011, Edagawa *et al*. 2008, Borella *et al.* 2004), where it causes a potential health risk for the people exposed. *Legionella* can be found free in the water phase, embedded in biofilm or intracellularly in amoebae (Van der kooij *et al.* 2005, Swanson & Hammer 2000). Some of the species most often found in water distribution systems are *Legionella pneumophila*, *Legionella anisa* and *Legionella dumoffi* but the last two mentioned is rarely implicated in human diseases (Gomez-Valero *et al* 2009, Pringler *et al* 2002).

1.1 Aim and approach

Because *Legionella* is widely distributed, it is not detection of the bacteria that is most important but the quantification of *Legionella*. *Legionella* has traditionally been detected by culture, but because culture depends on growth, detection can take up to 10 days. During an outbreak, days of waiting on an answer can make a substantial difference. The overall aim of the PhD was to improve the

background knowledge to accomplish risk assessment regarding *Legionella* in man-made water systems. One focus area was to validate the fast (2–3 hours) method quantitative real-time polymerase chain reaction (qPCR) for the detection of *Legionella* species, particularly *Legionella pneumophila*. Another aim was to test the validated qPCR on real-life water samples, to clarify if qPCR could be used in risk assessment and in monitoring during an outbreak. A newly built residential area was the main area for sampling because two Legionnaires' disease cases were associated with the area and the effect of two interventions could be followed.

In the thesis the focus was mainly on *Legionella* in habitations, but some parts of the discussion included water systems in general. Aims of this thesis have been to:

- Clarify how often *Legionella* is found in habitations.
- Evaluate the usability of qPCR in risk assessment in hot water systems.
- Address risk factors, including temperature, heating systems, physical/chemical factors, and other bacteria and amoebae.
- Describe the most important methods to overcome or prevent *Legionella* colonisation of water systems. Which methods are usable in immediate reactions and which methods can be used with a longer reaction perspective. The effect of permanent treatment of water is also addressed.

These points have been addressed based on own studies mainly conducted in the newly built residential area and based on a literature study.

2 Outbreak and clusters

In 1986, collaborators across Europe established The European Working Group for *Legionella* infections (EWGLI), and currently, 36 countries are members. Each year, each country is requested to submit a report with their annual dataset on Legionnaires' disease cases to the EWGLI database. Since 1994, epidemiological and microbiological trends within and between countries have been conducted based on the submitted information (Joseph & Ricketts 2010). A total of 5960 Legionnaires disease cases were reported in 2008. In the last 10 years the overall prevalence has been rising from 1442 cases in 1998 (Joseph & Ricketts 2010). The reasons for the increase in the number of reported cases are believed to be a mixture of more factors, increased knowledge about *Legionella*, better diagnostic methods, some large outbreaks and more countries participating in collecting data (Joseph & Ricketts 2010).

In 2008, 132 outbreaks or clusters were detected involving 391 cases (Joseph & Ricketts 2010). The terms 'outbreak' and 'cluster' have slightly different definitions according to different authors and situations. In this thesis the term 'outbreak' is used when more than two cases of Legionnaires' disease are correlated with the same source (often hospitals or cooling towers) over a short period of time (days or weeks). The term 'cluster' is used when more than one person is correlated with the same source. The timeframe between the cases in a cluster can be longer, from months to within two years. Clusters are often travel-associated. The above definitions also cover the descriptions behind the groupings used by Joseph & Ricketts 2010.

During 2007–2008 a total of 28 outbreaks involving 98 cases were linked to healthcare facilities and hospitals in Austria, Belgium, Cyprus, Denmark, England and Wales, France, Germany, Ireland, Italy, the Netherlands, Poland and Spain. In 22 of those instances, the source was found to be the hot or cold water system (Joseph & Ricketts 2010).

Since 2005, between 115 and 130 Legionnaires' disease cases have been reported each year in Denmark, from all over the country (Uldum *et al.* 2006-2010). In 2009, 116 cases were reported, 18 of which were fatal. This fatality rate of 15.5% is high compared to the average of 6.6% in Europe (in 2007 and 2008), based on reports to EWGLI (Joseph & Ricketts 2010).

Thirty-seven out of the 116 reported Danish cases were travel-associated, as those patients had been infected either travelling abroad or travelling in Denmark. Five were nosocomial cases, and for 74 cases the source of infection was in the category 'other' (Uldum *et al.* 2010). The category 'other' covers infections that occurred in the patient's own residence, which included 5 of these 74 cases (Uldum *et al.* 2010, Krøjgaard *et al.* 2011), and showers in public swimming pools, which included 2 cases (Uldum *et al.* 2010), but in most cases, 'other' is used because the source of the infection is not found

3 *Legionella* in an international and national perspective

3.1 Collection of samples

To clarify for later use, water samples are divided according to sampling time after opening the tap/shower hose, if appropriate and if such terminology was used in the cited publication:

- **A-sample** is the first amount of water collected when opening the tap, shower hose or drain from the heater tank.
- **B-sample** is water collected after some period of flushing.

This division of samples is used to clarify if the observed amount of *Legionella* could be due to local contamination, as in A-samples. In A-samples the water has been standing in the tap and the last part of the pipeline for an unknown amount of time. In B-samples, depending on the flushing time before collection of the sample, it could or should represent water from the circulation water system.

3.2 Legionella in hot water in habitations - an international perspective

To provide an overview of the dispersal of *Legionella* in habitations on an international scale, Table 1 has been compiled based on examples from the literature. Except from the study by Krøjgaard *et al* 2011 none of the surveys were associated with any outbreak or cluster of Legionnaires' disease

The prevalence of *Legionella* in B-samples from habitations was in the range of 6% to 32%. These numbers cover different sampling places, water tanks and taps as well as different sampling procedures. Some B-samples were sampled at constant water temperature, and some were tapped after a certain flushing. Furthermore, different media and different detection limits are used. Even with these uncertainties and inconsistencies, the table indicates that *Legionella* is quite often found in habitations and in fairly high amounts. In most studies, only hot water was collected because *Legionella* colonisation is rarely correlated to cold water (se chapter 3.2.2).

Table 1 The prevalence and amount of *Legionella* found in hot water systems in habitations on an international scale. Only the survey by Krøjgaard *et al* 2011 is conducted in relation to a cluster of Legionnaires' disease cases.

Author	Country	Sample collection site	No. of sample sites	No. of samples	A- or B-samples*	% of systems infected with *Legionella*	Concentration CFU /L
Krøjgaard *et al.* 2011	Denmark	Residential area	1	33	B	**	Positive samples: 5–5.5 * 10^4
Mathyes *et al.* 2008	Germany	Single-family homes	452	452	B	11	Average all: 3,4 * 10^2 – maximum 1 * 10^6
Leoni *et al.* 2005	Italy	Apartments	59	59	B	31	Positive samples: 25–9,8 * 10^4
Moore *et al.* 2006	USA	Buildings, homes	96	768	-	20/6 ***	Information not given
Borella *et al.* 2004	Italy	Homes	146	146	B	23	Average positive samples: 1,17 * 10^3 (range 25–8,7 * 10^4)
Marrie *et al.* 1994	Canada	Houses and apartments	90	225	-	11 ****	Information not given
Zacheus & Martikainen 1994	Finland	Apartments, few offices and small industries	67	201	A+B	30	Average positive samples: 2,7 * 10^3 (range < 50–3,2 * 10^5)
Stout *et al.* 1992	USA	Homes	218	654–1090	B	6	Positive samples: 1 * 10^4–6 * 10^5
Arary &Joly 1991	Canada	Private houses	211	970	A	33	Information not given
Arnow *et al.* 1985	USA	Houses and apartments	95	285	B	32	Positive samples: 1- 1 * 10^4 Median 2 *10^2

- * A-samples refer to samples tapped as first-flush samples, B-samples to samples tapped after the first flush.
- ** Samples were collected from different apartments in one residential area. They all had the same water circulation system.
- *** Samples were collected when chlorine was used as disinfectant (20 % colonisation with *Legionella*) and when monochloramine was used as disinfectant (6% colonisation with *Legionella*).
- **** A mix of hot and cold water was sampled.

Detection limits were rarely described.

3.2.1 Action requirements

To classify the amount found by cultivation into some action requirements, European guidelines have been published by members of the European Surveillance Scheme for Travel Associated Legionnaires' Disease and EGWLI members. See table 2. Here only the guidelines for warm and cold water systems are shown. A table and guidelines for cooling towers have also been published (Joseph *et al.* 2005).

Table 2. Action levels following *Legionella* sampling in hot and cold water systems. Table from European Guidelines for Control and Prevention of Travel Associated Legionnaires' disease (Joseph *et al.* 2005)

***Legionella* bacteria (CFU/litre)**	**Action required**
Alert: More than 1000 but less than 10.000	Either: (i) If only one or two samples are positive, system should be re-sampled. If a similar count is found again, then a review of the control measures and risk assessment should be carried out to identify anyremedial actions; (ii) If the majority of samples are positive, the system may be colonised, albeit at a low level, with *Legionella*. Disinfection of the system should be considered, but an immediate review of control measures and risk assessment should be carried out to identify any other remedial action required.
Action: More than 10.000	The system should be re-sampled and an immediate review of the control measures and risk assessment carried out to identify any remedial actions, including possible disinfection of the system.

The guidelines are, however, only guidelines, and different countries have different action values, which also could vary according to the sampling site (e.g., hospital, care home for elderly).

Denmark (Statens Serum Institut) has its own guidelines that are generally used (see table 3 below). The division of samples into A- and B-samples (as described above) is part of the guidelines. At Statens Serum Institut B-samples are tapped at constant temperature.

Table 3. The Danish reaction guidelines (in B-samples) when demonstrating *Legionella* in habitations in warm water systems (Albrechtsen *et al.* 2000).

Concentration of *Legionella* CFU/L	**Consequences for action**
10 to < 1000	Low amount, but shows that the conditions for growth of *Legionella* are present.
1000 to <10.000	Low to moderate amount. It should be considered whether simple improvements to the system could be conducted.
10.000–100.000	Relatively high amount. It should be considered whether improvements to the system and/or disinfection can be conducted. The situation should be followed.
≥100.000	Very high amount. The system should be examined with a view to reconstruction.

3.2.2 *Legionella* in water - a national perspective.

To set the foundation about the national surveys that have been conducted, the three largest studies on *Legionella* in different water systems are briefly described:

- In 1999–2000 *Legionella* was studied in different **public water systems** (schools, sport centres, etc.) (Hvidovre kommune 2001).
- In 2000 *Legionella* was studied in **private water systems** (residences) (Brydov *et al.* 2001).
- In 2002–2003 *Legionella* was investigated in **water works and water distribution networks** (Olsen *et al.* 2005).

Public water systems

A large survey was organised by the municipality of Hvidovre (2000) to investigate if and in what concentration *Legionella* existed in public water systems. The level of *Legionella* in hot water obtained from schools (12 water systems), sport centres (11 water systems), care homes for elderly people (5 water systems) and other buildings (4 water systems) was investigated (Hvidovre kommune, 2001). Mainly B-samples were collected, and culturing was the method of quantification.

The first round of water collection showed *Legionella* in 22 out of 24 water systems. In 13 cases the concentrations of *Legionella* caused reconstructions and thermal treatments. After treatments were implemented, water was collected

again, and then only 13 out of 32 water systems contained *Legionella*, and only three systems showed concentrations above 10^3 CFU/L. Further actions were initiated on those three water systems (Hvidovre kommune, 2001).

Residences

Residences have also been the focus for a large survey. Water samples were collected from 22 buildings, 13 of them residences (Brydov *et al.* 2001). Samples were collected as A-samples from the hot water tap or shower hose. Both cultivation and semi-quantitative PCR (the quantification was based on band intensity on gels, see chapter 4.4) were used to detect *Legionella pneumophila*. *Legionella* was found by cultivation in 12 of the 13 residences, and in 5 of them the amounts were above 10^4 CFU/L. By PCR, all 13 were positive, containing around 10^3 *Legionella pneumophila* Genome units (GU)/L or more.

Waterworks and distribution networks

The drinking water in Denmark is based on water extracted from ground water and normally has a high quality. To investigate if *Legionella* could be found in drinking water (cold water), 27 water works and 35 water distribution systems spread throughout Denmark were investigated (Olsen *et al.* 2005).

'Clean' water from water leaving the water works was collected together with biofilm scraps and filter material from those water works, where possible. The temperature of the water was below 12 °C. B-samples were collected from the distribution network from taps placed outside, taps routinely used to take control samples and from mixer taps of different consumers. The temperature in the distribution network was in the range 4–16° C.

None of the samples from the water works was colonised with *Legionella*. Only in two of the samples collected from the distribution networks was *Legionella* isolated, and only in very small concentrations (4 CFU/L and 40 CFU/L, respectively) (Olsen *et al.* 2005).

In Krøjgaard *et al.* (2011), cold water samples were also collected. These samples were from different apartments in a residential area. No *Legionella* was detected by culture (See table 8).

Legionella is not expected to be a problem when water is 'cold water' in the distribution network. It is in the warm water circulation systems that *Legionella* grows in numbers, or, as suggested by Arvand *et al.* (2011) based on a German study, when cold water gets heated. Arvand *et al.* 2011 conducted a survey on four health care facilities and often found *Legionella* in tapped cold water (collected from shower hoses and taps after discarding 3 L), but rarely in the distribution network. It is suggested that warming up effect could have happened if cold and warm pipes were closely fitted in the same shaft or because of long intervals of stasis. Another explanation is local contamination. Arvand *et al.* 2011 didn't find a correlation between water temperature and concentration of *Legionella* but explains that the warming up effect could have happened at a time when not measuring.

Summary

Both international and national studies showed a marked dispersal of *Legionella* in habitations. In international studies the prevalence was in the range from 6% to 32%. In Danish surveys thirteen out of 24 public hot water systems needed changes implemented to overcome the colonisation and five out of 13 samples from residences contained more than 10^4 *Legionella* CFU/L by culture. The samples from residences were, however, from the first flushes from taps and shower hoses, which could indicate a local colonisation from a specific tap/shower hose. In the hot water circulation system, the amounts were not necessarily as high. Colonisation of water systems is mainly found to be correlated to heated water.

4 Detection and quantification of *Legionella* in water

The two main methods to detect *Legionella* in water are culture and qPCR. Culture is the reference method, but qPCR is becoming more and more common (Krøjgaard *et al.* **II**, **III**, Bonetta *et al.* 2010, Joly *et al.* 2006, Yáñes *et al.* 2005, Wellinghausen *et al.* 2001, ISO 11731-2). Both methods are briefly described below, but because both methods depend on concentrated samples and qPCR also requires that the samples be purified, concentration and purification of water samples will first be addressed.

4.1 Concentration and purification

In Krøjgaard *et al.* 2011, **II** and **III**, concentration, purification and information about culturing the samples are explained. A short description will be given based on what is practised at Statens Serum Institut, but the same procedures for concentrating and culturing, or variations of these methods, are generally used (Bonetta *et al.* 2010, Leoni *et al.* 2005, Borella *et al.* 2004, Martinelli *et al.* 2000, Zacheus & Martikainen 1994, Arnow *et al.* 1985).

The stated method at Statens Serum Institut was applied in this work, and culture from water samples is conducted in three different concentration steps. The first step is direct seeding from water sample without any concentration. Then, water is filtered and the bacteria at the membranes are re-suspended in a small volume of sample water by vigorously shaking the membranes. Then a second seeding is conducted. From this concentration water is collected for later purification for testing by qPCR. The samples were further concentrated by centrifugation before the third seeding. The concentration of *Legionella* by culture, is based on the largest number of colonies from all three concentration steps.

Instead of loosening *Legionella* from the membrane by vigorously shaking, others rubs the filter by hand, use bacterium-binding beads or grow *Legionella* directly from the membrane placed on the medium (personal communication, Sandra Lai, Health protection Agency London, and Mathyes *et al*. 2008, Yáñes *et al.* 2005, States *et al.* 1987). Concentration and purification of water samples for qPCR are conducted at Statens Serum Institut by using the ion-exchange method called Chelex-100.

4.2 Culture

Generally, only three media are used to culture *Legionella*: BCYE, GVPC and MWY (Krøjgaard *et al.* 2011, **II**, **III**, Bonetta *et al.* 2010, Mathys *et al.* 2008, Leoni *et al.* 2005, Borella *et al.* 2004, Wellinghausen *et al.* 2001 Martinelli *et al.* 2000, Zacheus & Martikainen 1994). According to the ISO standard 11731-2, the basic BCYE medium and GVPC (selective antibiotic) medium are standard for culturing *Legionella.*

BCYE medium is composed of buffer/potassium hydroxide, ferric pyrophosphate, L-cysteine HCl and α-ketoglutarate. Buffer/potassium hydroxide maintains the pH and permits aerobic incubation. Ferric pyrophosphate and L-cysteine HCl represent indispensable nutritive elements for growth of *Legionella*, and α-ketoglutarate activates growth.

GVPC medium is named after the four antibiotics used: glycine, vancomycin, polymyxin and cycloheximide. Exchanging cycloheximide with another antibiotic, antisomycin, is the largest difference between GVPC medium and the third medium, called MWY (Modified Wadowsky Yee) (http://www.oxoid.com and Biokar diagnostics http: //www. solabia.fr; ISO11731-2).

At Statens Serum Institut, cultivation is conducted on MWY and GVPC media. These media are chosen based on the desire to inhibit growth of other water bacteria and because different *Legionella* species and strains grow more favourably on different media.

To inhibit growth of other bacteria, acid treatments and increased cultivation temperature can be implemented.

Positive and negative aspects of *Legionella* culture are listed in table 4

Table 4. Positive and negative aspects of culture as a method to measure the amount of *Legionella* in water.

Culture	
Positive	**Negative**
Only live and viable *Legionella* will be detected	*Legionella* not able to grow on medium will not be detected
Determination of types is based on a grown isolate	Takes one week to 10 days
CFU is given based on 3 concentration steps	Overgrowth by other water bacteria
	Overgrowth by *Legionella*
	Variable recovery rate

4.3 Genes and phylogeny

Five whole genomes of different strains of *Legionella pneumophila* strain Alcoy, Paris, Lens, Philadelphia and Corby has been sequenced and published (D´Auria *et al* 2010, Gomez-Valero *et al* 2009). Each of them contain a circular chromosome and strain Lens and Paris also a plasmid. The genome size is of 3.3-3.5 Mb and contain a high percentage of coding regions. The average length of the coding sequences is long and the genetic order is highly conserved among the five genomes (D´Auria *et al* 2010, Gomez-Valero et al 2009).

Different genes are used to differentiate on genus and species level; 16SrRMA and 5S are used to differentiate at genus level and at species level the gene macrophage infectiity potentiator (mip), rpoB or the hypervariable 23S-5S ribosomal intergenic spacer region are often used (Krøjgaard *et al* **II** , Gomez-Valero *et al* 2009).

4.4 Determination of types

To determine what type of *Legionella* is found in the water source, an often used method is the agglutination test. Cultured *Legionella* is mixed with antibodies from infected rabbits (supplied in a test kit). If agglutination is seen, an antibody–antigen reaction has taken place, clarifying that the unknown colony is in the same group as the antibody that caused agglutination. Oxoid produces a widely used kit (Krøjgaard et al. 2011, Bonetta *et al.* 2010, Mathys *et al.* 2008, Leoni *et al.* 2005, Borella *et al.* 2004). The kit has rabbit antibodies that cover and distinguish among *Legionella* species, *Legionella pneumophila* serogroup 1 and *Legionella pneumophila* serogroups 2–14.

Often, only an Oxoid test with a few colonies from each water sample is conducted, but if serogroups and subgroups should be further determined, the antibody–antigen method of monoclonal enzyme-linked immunosorbent assay (ELISA) is used. If strain determination is important, DNA sequencing is conducted.

4.5 PCR and qPCR

qPCR is a quantitative enhancement of PCR. PCR is a method to amplify a DNA sequence. Primers (artificially synthesised DNA sequences) locate the target area and a polymerase copies the target. The copied sequences can be visualised on a gel by colouring with ethidium bromide and viewing under ultraviolet light.

In the more advanced qPCR, the PCR products are quantified by comparing the amount of DNA or more precisely comparing the amplification rates in known standards with the amount (amplification rate) in the unknown samples. In qPCR, signals of amplification are given by probes. A probe is an artificially synthesised DNA sequence complementary to the target sequence and labelled with fluorescents molecules which are measured by the qPCR machine (Kubista *et al.* 2006, Mackay *et al.* 2002).

To compare the amount of DNA in standards and unknown samples, baseline and threshold lines are adjusted. A baseline setting has in these studies been chosen to be the first 3 to 15 cycles of amplification where little change in the fluorescence signal is observed. Amplification is happening exponential and a threshold line is manually placed in the part of the exponential growth phase where the signal from the standards exceeds the background "noise." The exact placement is decided by the largest slope of the exponential growth phase (highest r^2, linear regression C_t = ax, slobe =b, y-scale concentration log scale), and best fit of the standard curve (where the number of cycles between standards are the same). The point where each standard curve crosses the threshold is named the threshold cycle (C_t). The amount of DNA copies in the unknown samples is estimated by comparing the C_t values from the unknown samples to the C_t values from the standards with known amount of DNA (Dorak 2006).

Standards were produced from cultured harvest and Qiagen purified *Legionella pneumophila benidorm*. The amount of DNA in the standard was estimated based on comparisons with another portion, portion B of cultured harvest and density,

McFarland and counting chamber measured *Legionella pneumophila benidorm*. To verify for the amount of DNA in the produced standards, portion B was purified and also tested on qPCR. A comparison with the French DNA standard (SRM_LEGDNA_01 Legionelles centre National de Référence, Lyon, France) was conducted (Krøjgaard *et al.* **II**).

To asses the sensitivity in detecting all *Legionella* species and to investigate for cross-reactivity to bacteria of non-*Legionella* species, a selection of both *Legionella* species and non–*Legionella* bacteria were included in the study. All *Legionella* strains were seeded on BCYE agar plates and incubated before harvest. The other bacteria were cultured on adequate media. One colony for each strain was picked and purified with chelex. Each of the strains were tested by both the *Legionella* species and the *L. pneumophila* assay.

The limit of detection (LOD) and the limit of quantification (LOQ) for each assay were determined from ten replicates tested in the same run. LOD was defined as the lowest concentration where nine out of ten samples were found positive. LOQ was defined as the lowest number of copies allowing reliable quantification defined by a coefficient of variation ≤ 25%. LOQ was calculated based on BioRad's (California, USA) computer program according to the Afnor T90471 standard.

The production of standards, specificity, detection and quantification limits for both assays (*Legionella* species and *Legionella pneumophila*) for the qPCR used in Krøjgaard *et al.* **II** and **III** are further described in Krøjgaard *et al.* 2010.
Positive and negative aspects of using qPCR to measure *Legionella* are listed in table 5.

Table 5. Positive and negative aspects of qPCR as a method to measure the amount of *Legionella* in water

qPCR	
Positive	**Negative**
Detects all *Legionella*	Detects also dead *Legionella.*
Takes only 2–3 hours	Can only distinguish according to the target of the primers
Is quantitative	The amount in a sample is given after concentration and purification and only from one concentration step.

4.6 Comparisons of culture and qPCR in quantifying *Legionella* - usability in risk assessment

4.6.1 An overall correlation

Culture and qPCR with primers detecting *Legionella pneumophila* and *Legionella* species were compared in relation to risk assessments in Krøjgaard *et al.* **III**. Samples were collected from a newly built residential area to study the effect of two interventions (thermal treatment and hyperchlorination) on *Legionella* colonisation after an cluster with two cases of Legionnaires' disease. Both A- and B-samples were collected before, between and after the two interventions.

Overall, the amounts found by culture and by qPCR by both pairs of primers did not correlate well (r = 0.55, *Legionella* species assay and r =0.44, *Legionella pneumophila* assay). Weak correlations between culture and *Legionella* qPCR have also been found by others (r= 0.39, *Legionella pneumophila* assay, Bonetta *et al.* 2010 and r= 0.57, *Legionella pneumophila* assay, Wellinghausen *et al.* 2001).

Because qPCR amplifies both living and dead organisms' DNA, an effective intervention would not immediately change the amount of *Legionella* detected by qPCR, except for the DNA being destroyed during the intervention. As long as *Legionella* is in the water system, dead or alive, qPCR will quantify it. By culture, however, only the living and viable *Legionella* will be detected, which could partly explain the overall low correlation between the samples tested in Krøjgaard *et al.* **III.**

Lee *et al.* (2011) offered some additional explanations:

- The genome is duplicated before the cell divides, which could cause more genomes to be detected by qPCR than colonies detected by culture.
- Recovery from culture is between 10 and 60%.
- If the qPCR is not specific only for the target organism, either *Legionella pneumophila* or *Legionella* species, then other bacteria might be amplified and included in the measurements.

- Distribution of bacteria is not uniform. In the culture method, counting low numbers on medium does not imply a correct number.

Different recovery would cause difficulties when comparing culture with qPCR and even when comparing two cultured samples. The amount lost by concentration is unknown, and the cultivable amount out of the total amount is unknown. Optimisation of the process of concentrating water samples should be addressed and the variation in loss should be studied and improved.

The issues about specificity according to detection of non -*Legionella* species was addressed in the used assay, 35 non-*Legionella* bacteria species was tested and Genebank aligement and Primer-Express programme was used designing primers.

In Krøjgaard *et al.* **II**, 44 randomly collected samples from private residences, hospitals and cooling towers were tested by both culture and qPCR with both *Legionella* species and *Legionella pneumophila* primers. When studying correlations between culture and qPCR using the *Legionella* species assay, samples were divided into two groups. One group contained samples that showed no or only very limited amounts of *Legionella* by culture but were found to contain high concentrations by qPCR. With this group, no correlation analyses were conducted. The other group contained the rest of the samples, but here, the correlation was also weak (r= 0.36).

In contrast, when comparing culture results (no division in groups) and the amount found by the *Legionella pneumophila* qPCR assay, a good correlation was found (r = 0.77).

Lee *et al.* (2011), who used another qPCR *Legionella pneumophila* assay (Pall GeneSystems), also found a better correlation between culture and the *Legionella pneumophila* qPCR assay than between culture and the *Legionella* species qPCR assay (Pall GeneSystems). In hot and cold water samples (N=506), Lee *et al.* (2011) found a mean log difference for *Legionella* species and culture of 1.05. Between culture and the *Legionella pneumophila* qPCR assay, the mean log difference was only 0.62. The authors offer the medium as one reason for this difference. Because *Legionella pneumophila* is the species most commonly isolated from infected patients, the medium was originally developed to detect that species. The medium is not optimised to support growth of other/all

environmental species, which could affect the growth abilities of these other species.

To improve the usefulness of qPCR by implementing a method that ideally could differentiate between living and dead *Legionella*, Yanez *et al.* (2011) have suggested staining bacteria with propidium monoazide. Propidium monoazide should only penetrate membrane-compromised cells and thereby, after light exposure, make the DNA in those cells less likely to be amplified. Problems with propidium monoazide also penetrating live bacteria are not fully resolved, but propidium monoazide has been more selective to membrane-compromised bacteria than other dyes, such as ethidium monoazide.

4.6.2 Algorithm between culture and qPCR

To derive alert and action levels for *Legionella* qPCR in hot and cold water samples, an algorithm has been suggested based on culture and qPCR on 506 samples from hot and cold water (Lee *et al.* 2011). Based on the mean log difference found between the *Legionella pneumophila* qPCR assay (Pall GeneSystems) and culture, the amount found by the *Legionella pneumophila* assay was 4 times greater than the corresponding culture results. The alert limit was $4 * 10^3$ GU/L, and the action limit was $4 * 10^4$ GU/L. Using these guidelines, 69% of the tested samples would have entailed the same reaction from both types of assays: no action, alert or action; 4% would have caused completely different reactions. For the *Legionella* species assay, the alert/action limit was 10-fold the amount found for culture: $>10^4$ GU/L and $>10^5$ GU/L, respectively.

Lee *et al.* (2011) found the difference between culture and qPCR results to increase when temperature was rising. This increase would cause another mean log difference and, therefore, change the algorithm. The algorithm is therefore difficult to transform to the above-described samples from the residential area because of the use of interventions, including thermal treatments. The temperature of the 44 samples of mixed origin tested in Krøjgaard *et al* **II** was unknown and therefore the algorithm was not tested on them either.

4.6.3 qPCR used with specific samples

In Krøjgaard *et al.* **III**, samples were grouped according to collection origin: a) circulation water, b) first flush from empty apartments and c) first flush from

shower hoses. In samples collected from the circulating water and samples collected as first flushes from shower hoses, the same tendency according to the amount of *Legionella* was found using both culture and qPCR with both primers (see figures 1 and 2).

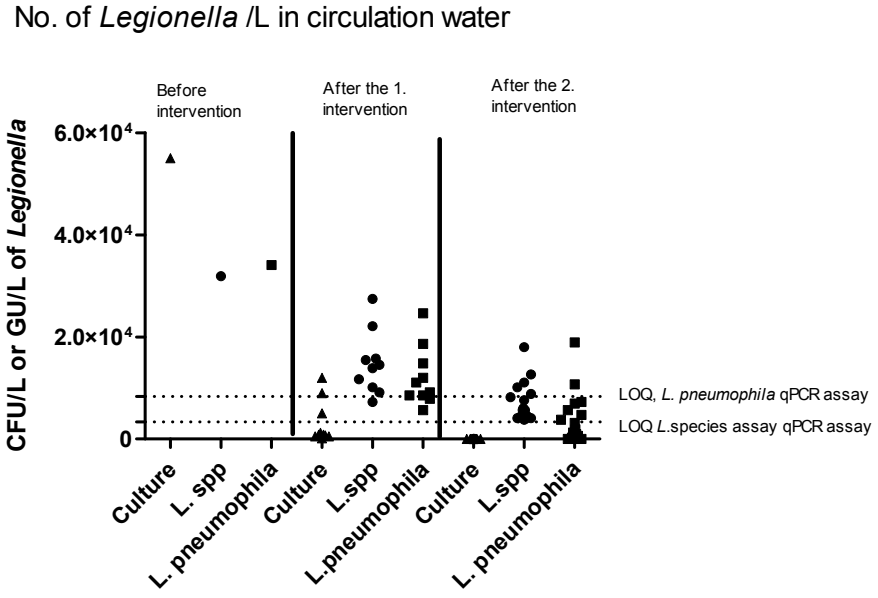

Figure 1. Comparison of the amount of *Legionella* detected by culture and by qPCR (both the *Legionella* species and the *Legionella pneumophila* assay) in circulation water before and after the two interventions. LOQ: Limit of quantification (Krøjgaard *et al.* **III**).

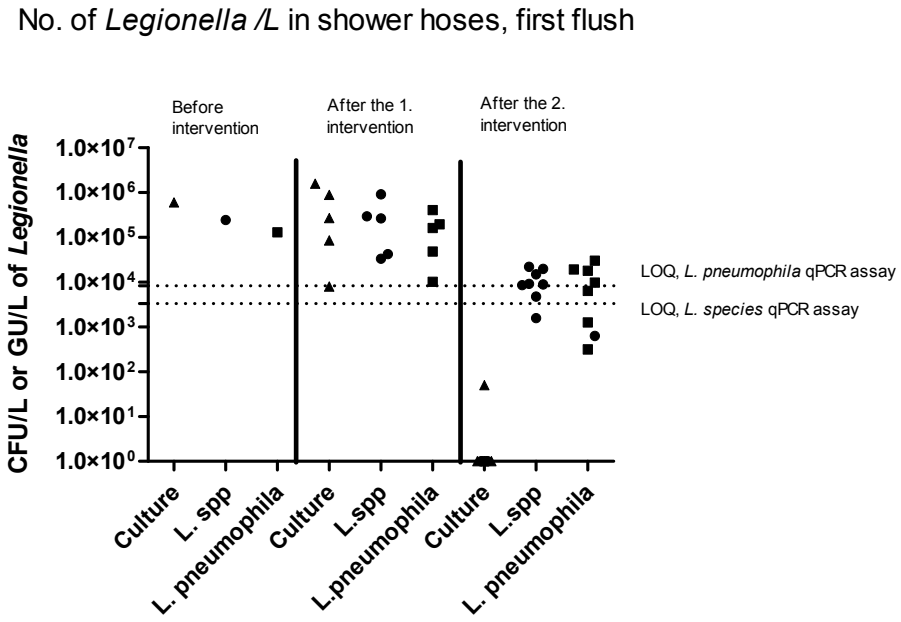

Figure 2. Comparison of the amount of *Legionella* detected by culture and by qPCR (both the *Legionella* species and the *Legionella pneumophila* assay) in the first flush of shower hoses before and after the two interventions. LOQ: Limit of quantification (Krøjgaard *et al.* **III**).

In samples collected from circulating water, the average amount of *Legionella* observed by both methods and by using both primer sets decreased after each thermal treatment. The amount of *Legionella* decreased in the first flush from shower hoses only after the second thermal treatment.

With a focus on these two groups of samples, qPCR was suitable when monitoring concentration developments over time. Because the ranges of *Legionella* numbers found by qPCR between and after the interventions often overlapped, it can be difficult to interpret distinct values.

Using the first flush from shower hoses, the amount of *Legionella* found by either method before the second intervention was very high. Water stagnancy and low temperature could cause shower hoses to become an important risk factor for *Legionella* growth.

In the samples collected as first flushes from empty apartments, samples were only collected before and after the second thermal treatment. Before the second thermal treatment, the amounts found by culture and qPCR were more or less in the same range (see figure 3).

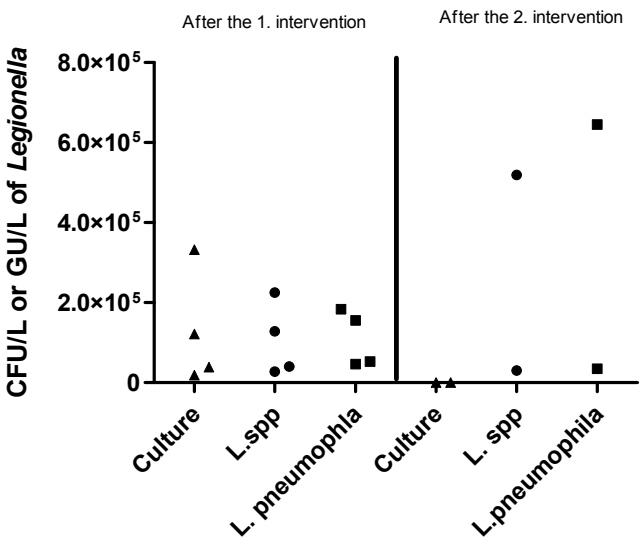

Figure 3. Comparison of the amount of *Legionella* detected by culture and by qPCR (both the *Legionella* species and the *Legionella pneumophila* assay) in empty-apartment first-flush samples before and after the second intervention.

After the second intervention, the high amounts ($5.2 * 10^5$ *Legionella* species GU/L and $6.5 * 10^5$ *Legionella pneumophila* GU/L) detected by qPCR must have been uncultivable or dead *Legionella* because only 10 CFU/L in one sample was found by culture. One explanation for these different amounts could be that the sample consisted of water that had been standing in the pipeline since the last thermal treatment or weekly flush. Weekly flush was initiated after the second thermal treatment in empty apartments. The temperature of the water used in thermal treatment or weekly flushing was able to kill *Legionella*. However a sudden opening of a tap could cause *Legionella* and biofilm to be flushed, and *Legionella* would thereby be detected by qPCR but not by culture.

In first-flush samples from empty apartments where thermal treatments/weekly flushing were conducted, qPCR was not ideal for risk assessment. It should be noted, however, that only two samples were collected after the second intervention, causing only a small background for generalisations.

A fundamental problem when comparing results from culture and from qPCR is that the amount obtained by culture is based on three concentration steps of the sample. In qPCR, only the second concentration step underlies further

purification and quantification. As shown in figure 1,2 and 3 the amount found by culture was sometimes higher than the amount quantified by qPCR. *Legionella* DNA was lost during the concentration and purification steps.

Summary

Both the culture and qPCR method have positive and negative aspects according detection and quantification of *Legionella*. Correlation of the amount given by each of the methods is not that simple and depend on temperature and potential treatment of the water system. When correlation culture and qPCR a algorithm was by others found between culture and qPCR pointing at a smaller difference between the amount detected by the *Legionella pneumophila* assay and culture than between culture and qPCR *Legionella* species assay. The correlation was weakened as the temperature rose.

Comparing specific samples on culture and qPCR the same tendencies was found for water tapped from the circulation system and from shower hoses but the distinct values given by qPCR are difficult to interpret.

5 Risk factors

The following chapter is based only on literature studies.

5.1 Temperature

An important and well-known risk factor for growth of *Legionella* is temperature. In the figure below, important temperatures for survival and growth of *Legionella* are depicted.

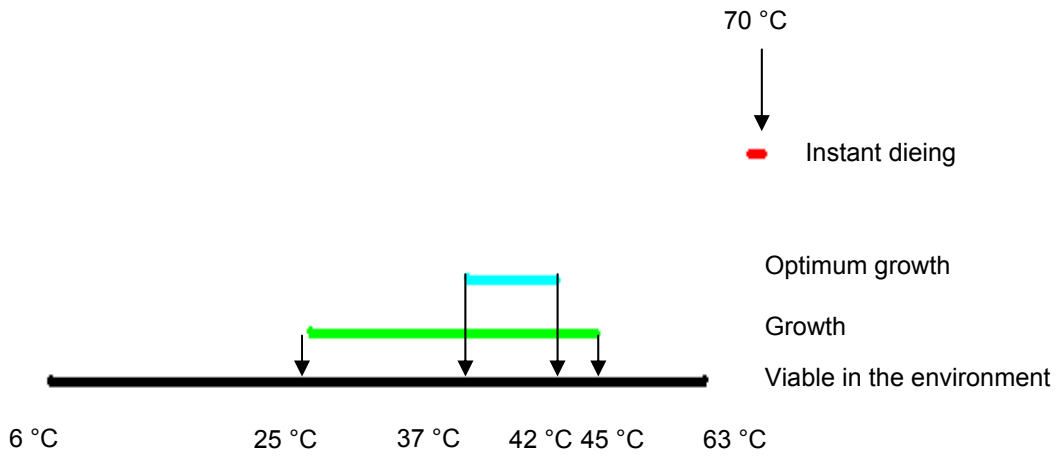

Figure 4. Important temperature for survival and growth of *Legionella*. Based on information from Bartram et al (2007), Yee & Wadowsky (1982), and Fliermans *et al.* (1981).

As water is not at all times in all parts of a circulation system at a temperature below 25 °C or above 45 °C, water systems can be colonised. In Krøjgaard *et al.* (2011), a B-sample after 15 minutes of flush (46 °C) showed 5.5 * 10^4 CFU/L. Martinelli *et al.* (2000) found a temperature dependency between faucet temperature and the frequency of isolated *Legionella*. If the water (N=107, A-samples) had a temperature ≤50 °C, 30% of samples were colonised with *Legionella*, whereas only 6.2% were colonised if the faucet temperature was >60 °C. The same tendency was observed in single-family homes (Mathys *et al.* 2008). Twelve percent of water samples with a temperature of 45.4 °C (lower quartile)–55.4 °C (upper quartile) (N= 400, B-samples) after flush were infected. No samples (N=52) with temperature quartiles of 52 °C–58 °C were colonised.

A study from Greece (Mouchtouri *et al.* 2007a) including 385 hotels (A- and B-samples, N=1086) showed that *Legionella* was not isolated from water with a

temperature below 23.7 °C or above 60.3 °C. Guidelines in Denmark regarding temperatures recommend that the whole water circulation system should operate at temperatures above 50 °C. Maintaining water temperature above 50 °C, even in the most distant tap and in the return water, is important. No parts of the circulation system should be vulnerable for colonisation (Albrechtsen *et al.* 2000).

Arnow *et al.* (1985) found culture-positive samples at 59 °C, and in Krøjgaard *et al.* 2011, *Legionella* were detected in water with a temperature of 57 °C. In the range 60 °C to 70 °C, it is difficult to determine at what temperature *Legionella* will certainly die. Exposure time and whether *Legionella* are exposed while free-floating, embedded in biofilm or situated in amoebae will make a difference. However, generally there is a negative correlation between temperature and colonisation. In the temperature range of 50 °C–60 °C, growth is limited.

5.2 Heater type

Regarding water temperature, different water heaters have been studied for their effects on *Legionella* colonisation. Table 6 summarises the results.

Table 6. The percentage of colonisation according to heater type. All results are based on culture.

Heater type	Authors	A-or B-samples	No. of samples tested	% colonised
Instantaneous supplies	Mathys *et al.* 2008	B-samples	52	0
	Martinelli *et al.* 2000	A-samples	64	6
Centralised heating	Leoni *et al.* 2005	B-samples	59	53
	Borella *et al.* 2004	B-samples	146	53 and 67 *
District heating	Mathys *et al.* 2008	B-samples	57	>50
Electric heaters	Borella *et a*l. 2004	B-samples	22	0
	Arary & Joly 1991	A-samples	178	37

*53% of single buildings and 67% of neighbourhoods, respectively

In samples from water systems using instantaneous supplies (tankless water heaters), the water was never or only rarely colonised. In the study where a few colonised samples were found (Martinelli *et al.* 2000), A-samples were collected. In the study where no samples were found to contain *Legionella*, B-samples were collected (Mathys *et al.* 2008). This differentiation in sampling procedure could explain the reported differences in colonisation, and the isolated *Legionella* could

be due to local contamination. The same explanation could perhaps be used as part of the explanation for the observed differences in electric heaters.

Samples from centralised heating and district heating systems were often (>50%) colonised. The temperatures of the tapped water in the different heating systems were given as one explanation by some authors (Mathys *et al.* 2008, Martinelli *et al.* 2000, Arary *et al.* 1991). Mathys *et al.* (2008) found the average temperature of instantaneous supplies to be 54.9 °C, whereas the temperature of distant heating systems was 47.9 ± 6.4 °C. Martinelli *et al.* (2000) always found instantaneous-supply water to be above 60 °C. The effect of heater type/the temperature of the water have an apparent importance according growth of *Legionella*.

5.3 Physical/chemical factors

5.3.1 Pipe material

Studies have investigated if specific material, e.g. pipe material, promote or inhibit growth of *Legionella*. Rogers *et al.* (1994a) tested eight different materials in a model system.

Table 7. Eight different materials were compared in a model system regarding the number of total flora and *Legionella* found in biofilm or the planktonic phase. Reproduced from Rogers *et al.*1994a.

		No. of microorganisms (mean)* in:	
Organism	**Material**	**Biofilm (CFU/ml * 10 ^{3})**	**Planktonic phase (CFU/ml *10 ^{3})**
Legionellae	Stainless steel	10.3	5.30
	Polypropylene	21.0	3.42
	PVCc	22.4	1.23
	PVCu	7.75	1.06
	Mild steel	20.6	5.30
	Polyethylene	6.76	6.68
	Ethylene-propylene	144	1.80
	Latex	220	13.8

* means were calculated from all values determined over 1 to 28 days.

Latex was the material supporting the highest amount of *Legionella* both in the biofilm and in the planktonic phase. Unplasticised polyvinyl chloride (PVCu) was the material that caused the smallest amount of *Legionella* in the planktonic phase and the second least amount in biofilm.

Copper was not one of the pipe materials tested by Rogers et al. (1994a), but other studies have been conducted on the effect of pipes made of this material. Van der *et al.* 2005 and Rogers *et al.* (1994b) found in model systems that copper limited the growth of *Legionella*. The survey of Rogers *et al.* (1994b) tested colonisation of *Legionella* at different temperatures (20 °C, 40 °C, 50 °C, 60 °C). The only temperature at which *Legionella* were isolated on copper pipes was 40 °C. The amount detected was a 35–50 times lower than the concentrations found on material made of PVCc or polybutylene. Van der Kooij *et al.* (2005) found *Legionella* in a concentration of 1500 CFU/L on pipes of copper and about 4300 CFU/L in pipes of stainless steel and cross-linked polyethylene. A limiting effect of copper when the content in the water phase was measured was found by Leoni *et al.* (2005), Borella *et al.* (2004) and Zacheus & Martikainen (1994).

One study conducted in single-family residences found, in contrast to the above findings, that copper pipelines were colonised more often and contained higher concentrations (17.1 % ± 37.8 SD, 609 ± 6432 CFU/100 mL± SD) than synthetic (2.7% ± 27.0 SD, 19 ± 151 CFU/100 mL ± SD) or galvanised-steel (3.3% ± 18. 2 SD, 1 ± 6 CFU/100 mL ± SD) pipelines (Mathys *et al.* 2008). No explanation was given, and the temperature of the water was similar in all three types of water systems.

Van der kooij *et al.* 2005, Rogers *et al.* 1994a and Rogers *et al.* 1994b all studied *Legionella pneumophila* but that was also the species most often isolated by Mathys *et al.* (2008).

It is difficult to determine the correct order of the extent of colonisation permitted by the various materials. Different materials are in turns found more colonised than other materials. Most studies though found a limiting effect of cobber.

5.3.2 pH

The potential correlations of pH and presence of *Legionella* have been studied. A higher pH was found in *Legionella pneumophila*/*Legionella* species–positive samples (pH 7,2 ± 0,3 S.D) than in *Legionella*-negative samples (pH 6,9 ± 0,3 S:D) (N = 137) (Leoni *et al.* 2004). The results from Mouchtouri *et al.* (2007a) agree with this correlation, but only for *Legionella pneumophila*. Other species were negatively correlated with pH.

5.4 The effects of water source and other bacteria

5.4.1 Water sources

The effects of using different water sources, specifically, ground water and surface water, on the amount of *Legionella* isolated have been studied (Borella *et al.* 2004, Zacheus & Martikanen 1994). Borella *et al.* (2004) (N=146) found no effect, whereas Zacheus & Martikanen (1994) did. Zacheus & Martikanen (1994) isolated *Legionella* from 12.5%, 29% and 37.5% of the hot water distribution systems (A- and B-samples, N= 201), which received, respectively, chlorinated groundwater, un-chlorinated groundwater and chlorinated surface water. The average concentration of *Legionella* in the water systems using surface water also was higher than in those systems receiving water from groundwater sources.

Zacheus & Martikanen (1994) also studied the microbiological characteristics in association with the origin of the water source. The content of total organic carbon was higher in systems receiving their water from surface-water plants (mean 7,3 mg C/L) than in those receiving water from groundwater plants (mean 5.6 mg C/L).Since surface water contains more total organic carbon it was suggested to play a role as a microbial substrate for *Legionella*, but the correlation was only slightly positive. Measuring accessible carbon was suggested as a more predictive method for the amount of bacteria (LeChevallier *et al.* 1991, Zacheus & Martikanen 1994).

5.4.2 Other bacteria

To test if other environmental organisms could exhibit syntrophy with *Legionella*, Stout *et al.* (1985) made use of the fact that cysteine is an essential nutrient for *Legionella* growth. *Legionella* and other environmental organisms were poured on BCYE agar lacking cysteine to study if *Legionella* could growth as satellite colonies (growth at the peripheral edge) around the environmental organisms. The appearance of *Legionella* would indicate nutritional symbiosis between *Legionella* and the organism. Satellite growth was observed in 16 out of 32 environmental organisms. Identification at the species level of the environmental organisms was often unsuccessful, but at the genus level, *Flavobacterium*, *Pseudomonas*, *Alcaligenes* and *Acinetobacter* were identified. Satellite growth experiments were also conducted by Wadowsky *et al.* (1985), and the results showed the same tendencies.

To study if environmental organisms could also affect the survival of *Legionella*, Stout *et al.* (1985) studied *Legionella* in sterile supernatant and in supernatant containing a mixed population of environmental organisms (the isolates described above). In sterile supernatant, the survival of *Legionella* was shorter than in supernatant, with a mixed population of environmental organisms. After 14 days, the amount of *Legionella* decreased from a little above 10^4 to around 10^3 CFU/ml, whereas *Legionella* incubated in sterile supernatant decreased to around zero.

Increased necrotrophic growth (growth based on dead organisms) of *Legionella* on high concentrations (10^8 to 10^9 CFU/6 ml) of *Pseudomonas putida*, *Escherichia coli*, *Acanthamoeba castellanii* and *Saccharomyces boulardii* has been observed (Temmerman *et al.* 2006). All *Legionella* concentrations were initially approximately 10^6 and, after necrotrophic growth, between $5.3*10^6$ CFU/ml and $1.1*10^7$ CFU/ml were observed. Tests of necrotrophic growth based on Gram-positive organisms did not have the same effect. The robust cell wall was given as a possible explanation. Also, living cells of *Pseudomonas putida* did not support growth.

Summary

In relation to water sources, one study did not find any differences in colonisation with *Legionella*. Another study isolated *Legionella* more often from water systems receiving their water from surface-water plants than in those receiving water from groundwater plants. The concentration of total carbon was higher in the former and was thought to be a substrate for *Legionella*. The correlation to *Legionella* was, however, only slightly positive, and accessible carbon was suggested as a better predictive measurement.

Environmental organisms can provide nutrients needed for *Legionella* in water. If *Legionella* use those nutrients to keep alive, it could explain the longer survival observed when *Legionella* was in a supernatant with environmental organisms than in sterilised supernatant alone. High concentrations of some species of dead Gram-negative organisms; fungi, bacteria and protozoa stimulated growth.

5.5 The effect of amoebae

5.5.1 Replication

More than 13 different species of amoebae have been found to host *Legionella* (Swanson and Hammer 2000). Surveys on the amplification of *Legionella* in amoeba have been conducted by, among others, Bouyer (2007), Shadrach *et al.* (2005), Storey *et al.* (2004), Abu (1996) and Holden *et al.* (1984). The life cycle of Acanthamoeba, an often used amoeba in studies of *Legionella* (Bouyer *et al.* 2007, Storey *et al.* 2004, Kilvington & Price 1990, Holden *et al.* 1984) occurs in two life stages: an actively feeding, dividing trophozoite and a dormant cyst that is more resistant to harsh environments (Marciano-Cabral & Cabral 2003).

Temmerman *et al.* (2006) compared, over a period of 14 days, growth of *Legionella* on heat-killed *Pseudomonas putida* with growth of *Legionella* when live *Acanthamoeba* were present. After replication associated with *Acanthamoeba*, $1.5 * 10^8 \pm 7{,}3 * 10^7$ *Legionella* GU/L was detected, whereas when only feeding on dead *Pseudomonas putida*, the amount was $6.2*10^5 \pm 2.1*10^5$ *Legionella* GU/L. However, during the first 96 hours, necrotrophy kept up with protozoon-mediated growth.

The studies of Shadrach *et al.* (2005), Abu (1996) and Holden *et al.* (1984) all studied the replication of *Legionella* in amoeba in a relatively short timeframe, <72 hours, and saw an increase in the amount of *Legionella*. Bouyer *et al.* (2007) and Storey *et al.* (2004) have studied growth and survival over longer periods, 180 days and 40 days, respectively, and they found different results. Bouyer *et al.* (2007) found long-lasting survival and growth. After 180 days, the amount of *Legionella* was increased from $1.2 * 10^3$ CFU /ml to $11 * 10^3$ CFU /ml (based on figure 1 in Boyer *et al.* 2007). Storey *et al.* (2004), in contrast, found a serious reduction of the amount of *Legionella* over time, from an average of 360 ± 72 CFU per trophozoite at day 1 to 7 ± 1 CFU at day 40. An intermediate–long study (growth in *Acanthamoeba polyphaga* trophozoites for 7 days) showed an increase in the amount of *Legionella* the first five days (from $9.5 * 10^5$ CFU/mL to $1.7 * 10^{11}$ CFU/mL), followed by a small decrease (Kilvington & Price 1990).

5.5.2 Protection against environmental treatments.

To investigate if *Legionella* is better protected against environmental factors when situated inside amoebae than free in the water phase, multiple experiments have been conducted. Storey *et al.* (2004) compared the effect of temperature

between *Legionella* in the planktonic phase and *Legionella* inside *Acanthamoeba castellanii* in the trophozoite state. As shown in figure 5, intracellular *Legionella* increased their resistance to thermal treatment 10- to 100-fold compared to the planktonic phase.

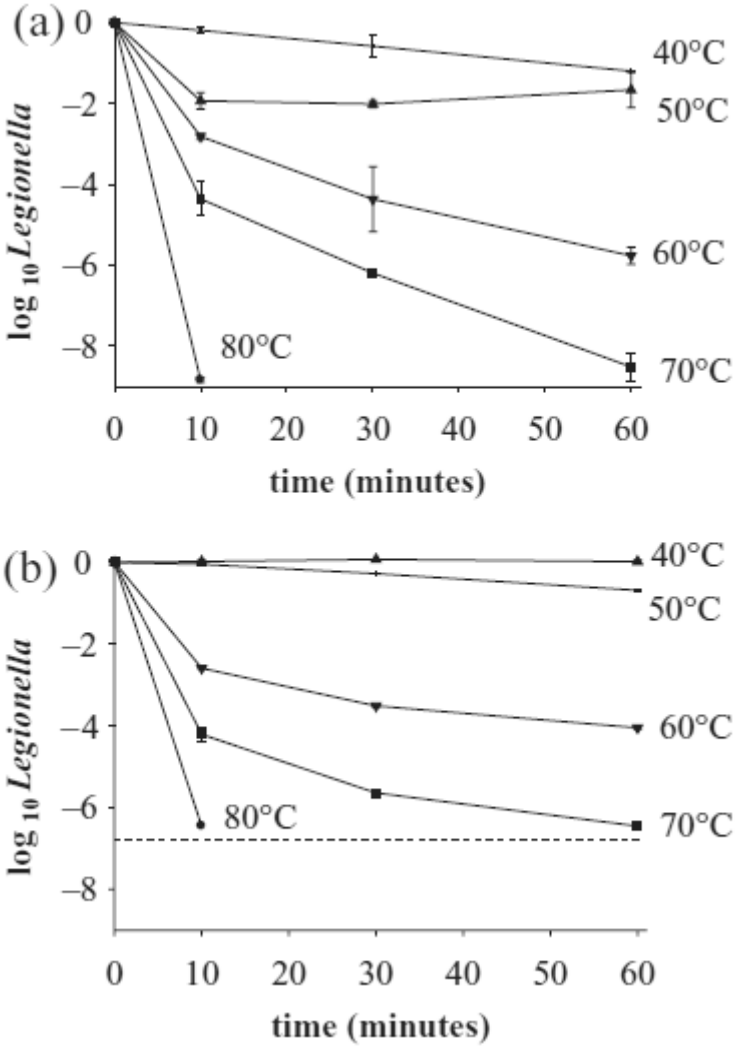

Figure 5. The efficiency of thermal treatments (°C) expressed as the log reduction of culturable a) planktonic and b) *A. castellanii*–bound *Legionella pneumophila* after 10, 30, and 60 minutes. Broken lines denote the limit of detection, and error bars represent 1 standard deviation (n=4) (Storey *et al.* 2004)

Concerning amoebae's role to protect *Legionella* towards chlorine, the results from two studies pointed in different directions. In the study by Storey *et al.* (2004), the effects of different concentrations of both free chlorine and combined chlorine were tested on both planktonic and *Acanthamoeba castellanii*–bound

Legionella. It was not clear whether trophozoites and/or cysts were used in this experiment. Conflicting with the thermal treatment study, the authors found that the amoebae did not protect *Legionella* very well. Only in low concentrations of free chlorine (1 mg/L) *Legionella* inside *Acanthamoeba castellanii* survived better than planktonic *Legionella*. Above 5 mg/l free and combined chlorine and more than 30 minutes of exposure, a reduction of 5 log was observed. The amount of *Legionella* was reduced to below the limit of detection.

A study by Kilvington & Price (1990) found, in contrast, a much higher degree of protection against free chlorine. The authors were able to grow *Legionella* from *Acanthamoeba polyphaga* cysts after 18 hours of exposure to 50 mg/l free chlorine. This concentration of chlorine is only used when conducting hyperchlorination.

Summary

Most studies showed that *Legionella* can amplify inside amoebae. Situated intracellularly in amoebae, *Legionella* is better protected against thermal treatments than when in the planktonic phase. Regarding chlorine use, the results from the two studies were inconclusive, which could have been because amoebae were used in different states, cysts or trophozoites, with cysts being more resistant to environmental factors. The change between cysts and trophozoites could also influence the amplification of *Legionella* in amoebae.

6 Treatments of hot water systems infected with *Legionella*

To overcome a *Legionella* colonisation or prevent further growth in a single or a few water circulation systems, methods such as thermal treatment, hyperchlorination, copper-silver ionisation and chlorine dioxide are used and will be discussed below. Thermal treatments and hyperchlorination are especially used if an immediate effect is wanted and both methods were conducted as part of the intervention in the main sampling area of this PhD "the newly built residential area".

Instead of disinfection limited to a single system, water works in many countries overcome bacterial growth by using chlorine in the distributed water. The use of monochloramine will be described regarding its effect on *Legionella*.

Methods like sodium hypochlorite, ultraviolet disinfection and point-of-use filters are also used to limit colonisation and exposure to *Legionella*, but they are not addressed in this thesis.

6.1 Thermal treatment

Because there are strict limits on additives in drinking water in Denmark, thermal treatment is an often used method to overcome a *Legionella* colonisation (Krøjgaard *et al.* 2011, Hvidovre Kommune 2001, https://www.retsinformation.dk/forms/R0710.

In Krøjgaard *et al.* (2011), the long-term effect of two thermal treatments, the second of them including a hyperchlorination, have been described. The study followed the effect of the treatments up to seven months after the second thermal treatment.

Before any interventions, a B-sample from the boiler (46 °C after 15 minutes of flush) revealed 5.5 * 10^4 *Legionella* CFU/L. One A-sample from a shower hose in the same apartment revealed more than 6 * 10^5 *Legionella* CFU/L.
The first thermal treatment lasted 12 hours at 70 °C in the boilers, and flushing of all taps was recommended. The flow in the water system was raised. Until the second thermal treatment three weeks later, the boiler temperature was 60 °C.

Because the amount of *Legionella* did not decrease sufficiently (see figures 6 and 7 and table 8), the second thermal treatment was conducted.

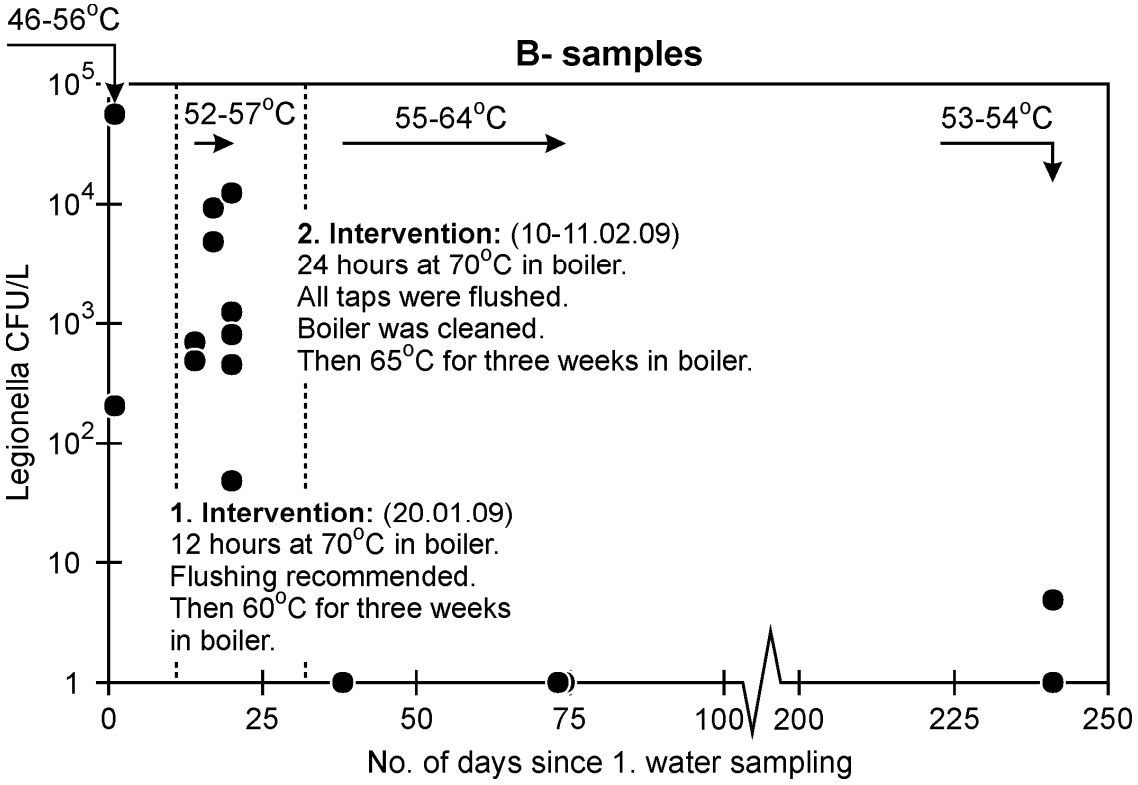

Figure 6. Concentration of *Legionella* spp. over time in the hot circulation water in the block of flats. One-litre water samples were collected after flushing until constant temperature (B-samples), and the temperature interval in the samples in the different apartments are given at the top of the figure. Each dot represents one sample, but there are two samples with 800 CFU/L on day 20, six samples with no *Legionella* detected on day 38, four samples with no *Legionella* detected on day 73, four samples with no *Legionella* detected on day 74 and five samples with no *Legionella* detected on day 241. The dotted lines indicate the first and the second interventions, respectively. The first water samples were collected on 9 January 2009. CFU: colony-forming units.

Source*:* Krøjgaard LH, Krogfelt KA, Albrechtsen HJ, Uldum SA. Cluster of Legionnaires' disease in a newly built block of flats, Denmark, December 2008 – January 2009. Euro Surveill. 2011;16(1):pii=19759.

Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19759

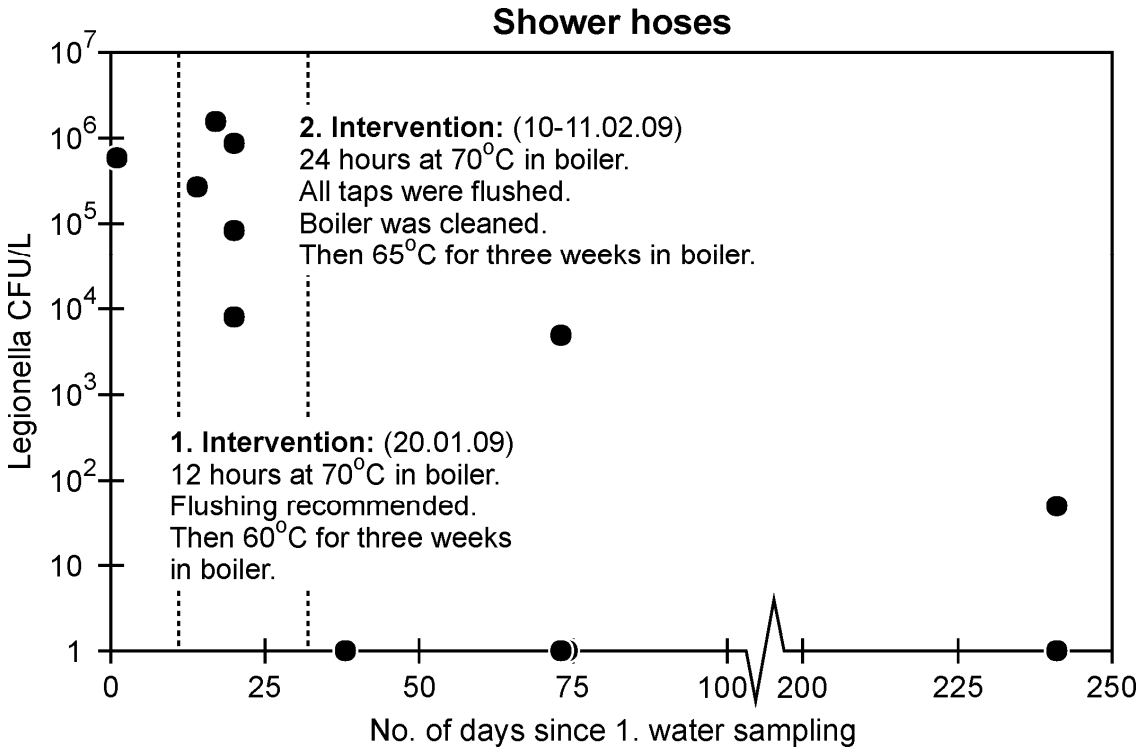

Figure 7. Concentration of *Legionella* spp. over time in the first litre of water sampled (A-samples) from different shower hoses. There are three samples on day 38, in which no *Legionella* could be detected. Each dot represents one sample, but there are two samples on day 73 and one sample on day 74 in which no *Legionella* could be detected. At day 241, there are two samples with no *Legionella* detected. The dotted lines indicate the first and the second interventions, respectively. The first water samples were collected 9 January 2009.

Source: Krøjgaard LH, Krogfelt KA, Albrechtsen HJ, Uldum SA. Cluster of Legionnaires' disease in a newly built block of flats, Denmark, December 2008 – January 2009. Euro Surveill. 2011;16(1):pii=19759.

Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19759

Table 8. Effect of interventions on the number and species, serogroups, strains of *Legionella*, data collected from seven different apartments, Copenhagen, January-September

Source: Krøjgaard LH, Krogfelt KA, Albrechtsen HJ, Uldum SA. Cluster of Legionnaires' disease in a newly built block of flats, Denmark, December 2008 – January 2009. Euro Surveill. 2011;16(1):pii=19759. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19759

Timing of the sampling	Sampling site	Type of sample[a]	Number of samples	Number of positive samples	Temperature of water tested (°C)	*Legionella* concentration CFU/litre CFU/L Median		Type of *Legionella* identified
Before the first intervention (9/1/09)	Shower hose	A	1	1	not measured	$> 6 * 10^5$	–	*L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3
	Tap	A	1	1	not measured	$1{,}4 * 10^5$	–	*L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3
	Tap (hot water)	B	1	1	46	$5{,}5 * 10^4$	–	*L. pneumophila* serogroup 1 subgroup Philadelphia
	Kitchen tap (hot water)	B	1	1	56	$2{,}0 * 10^2$	–	Sg 3
After the first intervention	Shower hose	A	5	5	not measured	$8{,}0 * 10^2 – 1{,}6 * 10^6$	266000	Sg 1. *L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3. Sg 2-14. Sg 4 subgroup Portland.
	Shower hose 38 °C[b]	B	4	4		$2{,}0 * 10^2 – 1{,}2 * 10^4$	9000	Sg 1. *L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3. Sg 2-14
	Bathroom tap (hot water)	A	5	5	not measured	$5{,}0 * 10^3 – 1{,}2 * 10^5$	20000	Sg 1. *L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3. Sg 2-14
	Bathroom tap (hot water)	B	5	5	51.5–56	$4{,}5 * 1+ ^2 – 1{,}2 * 10^4$	800	*L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3. Sg 2-14.
	Kitchen tap (hot water)	A	5	5	not measured	$7 * 10^2 – 3{,}3 * 10^5$	31000	*L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3. Sg 2-14
	Kitchen tap (hot water)	B	5	5	52–57	$5 * 10^1 – 5 * 10^3$	800	Sg 3. Sg 2-14
	Bathroom tap (cold water)	B	4	0	8.5–16	BD	BD	–
After the second intervention	Shower hose	A	7	1	not measured	$BD – 5 * 10^3$	BD	spp. *L. anisa*
	Shower hose 38 °C[b]	B	7	3		$BD – 1 * 10^2$	BD	*L. anisa*
	Bathroom tap (hot water)	A	7	0	not measured	BD	BD	–
	Bathroom tap (hot water)	B	7	0	55.3–64	BD	BD	–
	Kitchen tap (hot water)	A	7	3	not measured	$BD – 1 * 10^3$	BD	spp. *L. anisa*
	Kitchen tap (hot water)	B	7	0	56.7–64	BD	BD	–
	Bathroom tap (cold water)	B	3	0	7.3–16.7	BD	BD	–
Seven months after the second intervention	Shower hose	A	3	1	not measured	$BD – 5 * 10^1$	BD	Sg 3. Sg 2-14
	Shower hose 38 °C[b]	B	3	2		$BD – 1 * 10^2$	5	spp.
	Bathroom tap (hot water)	A	3	2	not measured	$BD – 2 * 10^1$	5	*L. pneumophila* serogroup 1 subgroup Philadelphia. Sg 3. Sg 2-14
	Bathroom tap (hot water)	B	3	0	53–54	BD	BD	–
	Kitchen tap (hot water)	A	3	1	not measured	$BD – 5 * 10^1$	BD	Sg 3. Sg 2-14
	Kitchen tap (hot water)	B	3	1	54	BD – 5	BD	Sg 3

BD: below detection by culture; CFU: colony-forming unit; Sg: serogroup.

[a] A-samples are the first litre of water from the tap or shower hose (first flush); B-samples are one-litre samples collected after flushing to reach constant water temperature (warm or cold) was reached.

[b] Samples of taps are collected after flushing until constant temperature. B-samples from shower hoses were collected when the thermostats were mixing cold and warm water to 38 °C. If B-samples are not referred to in the text as being from shower hoses, B-samples are from samples collected at constant temperature from taps.

The second thermal treatment was initiated by 24 hours, with boiler water temperature at 70 °C. Thereafter, boiler water was 65 °C for 3 weeks. All taps were flushed for 5 minutes, and the temperature in taps was a minimum of 65 °C. As part of the processes conducted to overcome the *Legionella* colonisation, boilers were hyperchlorinated and shower hoses were replaced with new ones. All of these changes will together be referred to as the second intervention. The water temperature in the whole system was followed closely in the following months, and boiler water temperature was slowly lowered. While lowering boiling water temperature, water from taps was kept at or above 50 °C. Constantly running water circulation systems at a substantially increased temperature would cause enormous energy consumption and increased corrosion.

As shown in figures 6 and 7 and table 8, the amount of *Legionella* decreased drastically after the second intervention, and measurements 7 months after showed no or only low ($\leq 10^2$ CFU/L) numbers of *Legionella*.

The low number of *Legionella* detected immediately after the second intervention (table 8) were all *Legionella anisa*, and they seemed to be able to survive higher temperatures than *Legionella pneumophila*. Krøjgaard *et al.* (2011) isolated seven months after the second intervention, the same serogroup (*Legionella pneumophila* serogroup 3) and subgroups (*Legionella pneumophila* serogroup 1 subgroup Philadelphia) as before the second intervention (table 8). This could indicate that *Legionella* either survived in the biofilm or that the same subgroup entered that water supply multiple times, which seems unlikely because it is rarely found in hot water systems in Denmark (Pringler *et al.* 2002).

Other studies (Farhat *et al.* 2010, Saby *et al.* 2005) support the idea that *Legionella* survives in the biofilm after thermal treatments. Farhat *et al.* (2010) observed fast re-colonisation on a pilot scale system both after the first and after the second thermal treatment (30 min of 70 °C hot water). The initial concentration of cultivable *Legionella* was 10^5 CFU/L. Twenty-four hours after the first 30 min thermal treatment, no *Legionella* were detected, but 48 hours after, 10^3 *Legionella* CFU/L were found, and the number increased to $6.2 * 10^4$ after a week. The second thermal treatment increased the number of *Legionella*, and thereafter, the amount was the same as before for the rest of the measuring period (7 days).

Mouchtouri *et al.* (2007b) studied the effect of longer thermal treatments (70–80 °C, 2–3 days and flush) on the amount of *Legionella.* Water was collected from water circulation systems from hotels, hospitals and athletic venues. Samples (A- and B-samples) were collected two to seven days after each of the thermal treatments.

In contrast to Krøjgaard *et al.* (2011), Mouchtouri *et al.* (2007b) observed a marked fall in the amount of isolated *Legionella* already after the first thermal treatment. Still, after the second treatment, few samples were positive ($\leq 10^4$ CFU /L).

Summary
The effect of thermal treatment depends on the duration and follow-up changes in the whole water system. Short (30 min) thermal treatments are not effective when measuring the effect over days. Longer treatments (1–3 days followed by generally higher circulation water temperature) are better to control the *Legionella* colonisation. Seven months after thermal treatments, the amount of *Legionella* was still found low. The small amounts of *Legionella* isolated after the thermal treatments, and especially the fact that the same subgroup was detected before and after the thermal treatments, suggest a risk of re-growth if the whole water circulation system is not strictly maintained.

6.2 Hyperchlorination

Chlorine can be added to water in gaseous or liquid form, and the effect on bacteria is alteration of respiratory and transport activities (http://water.epa.gov) and Kim *et al.* 2002).

Shock hyperchlorination (periodic free residual chlorination at a dosage of 20–50 mg/L) is used to clean of water tanks or whole water systems. Contact time depends on the concentration of chlorine, but at least two hours with 20 mg/L of chlorine or one hour with 50 mg/L is needed. A temperature below 30 °C is recommended. After chlorination, fresh water is let into the system before use (Joseph *et al.* 2005).

A long-term study of repeated hyperchlorination at three different water systems, a hospital (N=186 samples), a fishing boat (N=60 samples) and a hotel (N=57 samples), was conducted by Garcia *et al.* (2008) to observe the effect on

Legionella colonisation. The three facilities were all associated with Legionnaires' disease cases, and all of them underwent a number of hyperchlorinations during the study period of 5 to 17 years. No information about the samples (A- or B-samples) was given. Although no *Legionella* was detected after each treatment, all water systems were re-colonised after a few months, and the same strains of *Legionella* persisted in each installation throughout the study period. Hyperchlorination did not solve the persistence of *Legionella.* Because the daily operational methods of the water systems were not appropriate, re-colonisation occurred.

Hyperchlorination of boilers was also conducted by Krøjgaard *et al.* (2011) as part of the process to overcome *Legionella* colonisation. In that system, *Legionella* levels were subsequently kept very low but the maintaining temperatures was also altered.

6.3 Copper-silver treatment

Another method used to control colonisation of *Legionella* in a single or a few water circulation systems utilises the interaction between negatively charged cellular membranes and positively charged copper-silver ions. By channelling water through a device that applies low potential electricity to copper and silver electrodes, positively charged ions are fed into the water. The ions form electrostatic unions with the negatively charged cellular membranes, which alter the permeability of the cell membranes. Permeability changes will cause denaturation of proteins and subsequent cellular lysis (Cachafeiro *et al.* 2007, Stout & Yu 2003).

The level of ions should be in a certain range for optimal efficiency. Recommended levels in the review of Cachafeiro *et al.* (2007) are between 0.2 mg/L and 0.4 mg/L for copper and between 0.02 mg/L and 0.04 mg/L for silver. These recommendations can vary with water quality. The maximum recommended level of copper in drinking water is 2 mg/L, and 0.1 mg/L of silver could be tolerated (World Health Organisation. Copper, 2011 World Health Organisation, Silver 2011)

A large and long-standing survey on the effect of copper-silver ionisation at hospitals has been conducted (Stout & Yu 2003). The amount of *Legionella* in 16 hospitals was followed before and after the installation of copper-silver

ionisation systems in a period up to 11 years. No information is given about collection of either A- or B- samples. Prior to the installation, in 7 out of 15 hospitals more than 30% of samples were positive for *Legionella*. One hospital did not give that information. All hospitals reported nosocomially acquired Legionnaires' disease. Before installation of copper-silver electrodes, 75% of hospitals had conducted different disinfection treatments (thermal treatments, chlorine and ultraviolet radiation alone or in combination). After 5 to 11 years of operation, 7 out of 16 hospitals reported that *Legionella* was not detected in their water systems, and no hospitals found more than 30% of their samples colonised. Fifteen out of the 16 hospitals did not report nosocomial Legionnaires' disease. The one hospital that did reported only one case, and that was soon after the installation of the copper-silver treatment.

Another survey investigating the effect of copper-silver ionisation also found an apparent decrease in *Legionella* positive samples after implementation (Liu *et al.* 1998).

Based on the above-described surveys and supported by a review by Cachafeiro *et al.* (2007), copper-silver treatment is an effective method to control *Legionella* in water systems. Still, this method alone cannot completely eradiate *Legionella* in most systems, and to function optimally, the copper-silver installation must be maintained.

In Denmark, the copper-silver method is not used because the limits of copper and silver at the entrance of a building are lower respectively 0,1 mg/L and 0,01 mg/L, than the concentrations used for effective copper-silver treatments: (https://www.retsinformation.dk/forms/R0710).

6.4 Chlorine dioxide

Chlorine dioxide is another compound used against colonisation with *Legionella* in water circulation systems. Chlorine dioxide is a gas generated mechanically or electrolytically from sodium chlorite solution, and it kills bacteria by disrupting the transport of nutrients across the cell wall and disrupting protein synthesis (Kim *et al.* 2002).

Using chlorine dioxide to disinfect portable water is a more widely used method in Europe than in the US. In Denmark, however, there has been no continuous

use of chlorine in drinking water since 2009 (http://www.dr.dk/Nyheder/Indland/2009/06, Kim *et al.* 2002). The United States Environmental Protection Agency recommends that the chlorine dioxide concentration not exceed 0,8 mg/L (http://water.epa.gov).

The effect of using chlorine dioxide as disinfectant in a hospital's hot water system on *Legionella* colonisation has been studied (Zhang *et al.* 2007). A-samples were obtained for *Legionella* detection, whereas B-samples were obtained for chlorine dioxide analysis. Water was collected seven months and 30 months after installation. Chlorine dioxide was injected into the cold water main. The concentration of residual chlorine dioxide rose from 0.04 mg/L to 0.11 mg/L during the 30 months. During the same time, the number of *Legionella*-positive samples decreased from 60% (12 out of 20 samples) to 10% (2 out of 20 samples). Unexpectedly, fluctuations in the number of positive samples were observed (see figure 9).

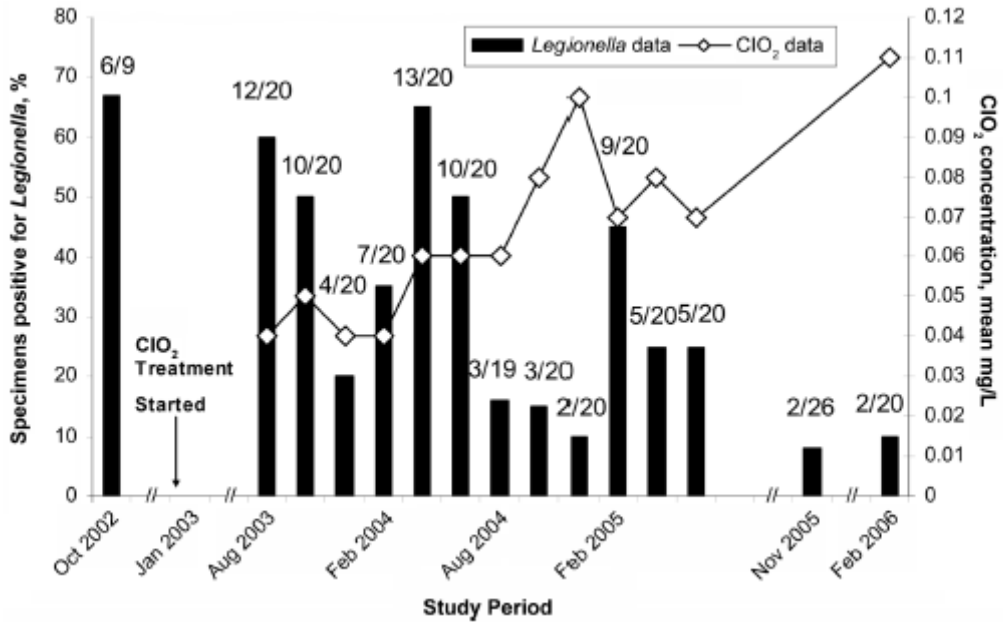

Figure 9. Percentage of samples positive for *Legionella* and concentrations of chlorine dioxide in hot water samples. The rations denote the number of *Legionella* positive hot water samples/ number of samples tested (Zhang *et al.* 2007).

Overall the number of positive samples decreased, but the concentration of *Legionella* in the positive samples remained constant. Chlorine dioxide ≥0.10 mg/L was the most effective at limiting *Legionella* in water.

A study conducted by Srinivasan *et al.* (2003) at another hospital, also using chlorine dioxide to control *Legionella* colonisation, found that chlorine dioxide had a limiting effect on *Legionella*. During the 17-month survey, the number of positive samples (B-samples) decreased from 41% when the use of chlorine dioxide was initiated to 4% at the end of the survey.

The number of positive samples was reduced after implementation of chlorine dioxide treatment, but at one of the hospitals, serious fluctuations were observed. A-samples were collected at that hospital, which suggests local contamination as part of the explanation.

6.5 Monochloramine

Due to concerns about harmful by-products, such as trihalomethanes, when using only chlorine, ammonia was added to chlorinated water to produce monochloramine. The effect on *Legionella* was studied (Flannery *et al.* 2006, Moore *et al.* 2006 & Kim *et al.* 2002). Monochloramine has been used as a continuous disinfectant in potable water systems, where it remains active for a longer time than chlorine and reacts less often with organic matter. It affects bacteria by penetrating the cell wall and blocking metabolism.

Three compounds are formed when chlorine and ammonia react: monochloramine, dichloramine and trichloramine. The three compounds have different optimum pH, and above pH 7, mostly monochloramine is formed. The United States Environmental Protection Agency recommends that chloramine concentrations not exceed 4.0 mg/L.

The effect of using monochloramine instead of chorine on *Legionella* colonisation was studied by Moore *et al.* (2006). B-samples were collected from 96 buildings during a time period when chlorine was the primary residual disinfectant and from the same buildings after monochloramine was introduced into the municipal water system. When chlorine was used as disinfectant, 19.8% of the buildings were colonised with *Legionella*. That number fell to 6.2% after the shift to monochloramine as the disinfectant. The same tendency was found in a survey of 53 buildings by Flannery *et al.* (2006). They found that 70% of hot water systems were colonised while chlorine was used by the municipal water system, which decreased to 9% after changing to monochloramine. Mostly B-samples were collected.

The use of monochloramine in potable water instead of chlorine has a limiting effect on the colonisation of *Legionella* in water systems.

7 Conclusion

Legionella are widely distributed and are often isolated in habitations all over the world. The percentage of colonisation of *Legionella* in residences are found in the range from 6% to 33%. Both culture and qPCR are used to quantify *Legionella* in water samples.

Emphasis of my work has been on clarifying if the newly validated qPCR targeting *Legionella* species or *Legionella pneumophila* could be used in risk assessment. Based on a comparison between culture and qPCR the usability of qPCR in risk assessment has been studied.

- The overall correlation between culture and qPCR is low when thermal treatments have been applied to water systems and boilers have been hyperchlorinated.
- Grouping samples according to their type and how they were collected e.g. ‘circulation water’ and ‘water from first flush from shower hoses’, culture and qPCR showed the same tendencies.
- It is difficult to interpret the distinct amount given by qPCR due to overlap in measurements before and after treatments.
- Comparing qPCR with culture on first flushes from empty apartments was inconclusive.
- In samples collected randomly from hospitals, private residents, schools and cooling towers, the *Legionella pneumophila* qPCR assay showed good correlation with culture.
- Information about the operation of the water system is important when interpreting qPCR results.

Another aim of this PhD thesis was to provide an overview of some important risk factors for *Legionella* colonisation in man-made hot water systems associated with habitations based mainly on literature studies.

- *Legionella* does not seem to be a problem in cold water.
- Water from circulation systems using centralised heating and distinct heating systems are more (>50% of samples) often colonised than water collected from water systems using instantaneous heating (heating without a storage tank) (0-6%).
- Temperature insufficient to control *Legionella* growth in the whole circulation system is probably the most important risk factor.
- Other environmental organisms, increases survival of *Legionella*. Dead Gram-negative organisms in high concentrations can promote *Legionella* growth.
- In amoebae, amplification of *Legionella* can take place, and whilst situated in amoebae, *Legionella* is better protected against thermal treatments.
- Copper pipes are usually found to have an inhibitory effect on colonisation, and pH is usually slightly higher in *Legionella*-positive samples than in samples where *Legionella* are not isolated.

Another aim of this thesis was to discuss some of the methods used to overcome *Legionella* colonisation both based on own experiments and literature. My studies showed that

- Thermal treatment initiated with 24 hours 70°C in the boilers, flush, hyperchlorination of the boilers and increased operational temperature in the whole water system are effective at rapidly reducing *Legionella*.

Other surveys found that hyperchlorination alone with no changes in the daily operation was not effective. Copper-silver ionisation and chlorine dioxide are generally found effective in reducing colonisation of *Legionella*. Treating the water system permanently with monochloramine a decreases in the amount of *Legionella* was detected when comparing to treatment with chlorine alone. These last mentioned treatments methods imply additives to drinking water which is not practice in Denmark.

Outlook

In future method-based work, it will be important to incorporate and optimise a method such as colouring of membrane-compromised cells. This approach would substantially improve the usability of qPCR in risk assessment because only live cells would be amplified. Another focus area should be to improve concentration methods for water samples because this is fundamental for accurate measurements.

8 References

Abu, K. Y. 1996. The phagosome containing *Legionella* pneumophila within the protozoan Hartmannella vermiformis is surrounded by the rough endoplasmic reticulum. Appl. Environ. Microbiol. **62**:2022-2028.

Alary M. and J.R. Joly. 1991. Risk factors for contamination of domestic hot water systems by *Legionella* Appl. Environ. Microbiol **57**: 2360-2367.

Albrechtsen, H-J., L. Bagge, L. Bagh, S. Bang, E.T.Jensen, O.B. Jepsen,M.S. Nielsen, C. R. Petersen, A-M. Plesner, M. Søeby, A.S.Thomsen and S.Uldum. 2000. *Legionella* I varmt brugsvand. Overvågning, udredning og forebyggelse af Legionærsygdom. Den centrale afdeling for sygehushygiejen Statens Serum Institut. 1 udgave.

Arnow, P. M., D. Weil, and M. F. Para. 1985. Prevalence and significance of *Legionella* pneumophila contamination of residential hot-tap water systems. J. Infect. Dis. **152**:145-151.

Arvand M., K.Jungkind, A Hack. 2011. Contamination of the cold water distribution system of health care facilities by *Legionella* pneumophila: Do we know the true dimension? Euro Surveill.:16:pii=19844.

Bartram J., Y. Chartier, JV. Lee, K., Pond, S. Surman-Lee. 2007. In: *Legionella* and the prevention of legionellosis. WHO (world health organization), WHO Library Cataloguing -in-Publication Data Geneva Switzerland ISBN 92 4 156297 pp 1-23.

Bonetta, S., S. Bonetta, E. Ferretti, F. Balocco, and E. Carraro. 2010. Evaluation of *Legionella* pneumophila contamination in Italian hotel water systems by quantitative real-time PCR and culture methods. J. Appl. Microbiol. **108**:1576-1583.

Borella, P., M. T. Montagna, V. Romano-Spica, S. Stampi, G. Stancanelli, M. Triassi, R. Neglia, I. Marchesi, G. Fantuzzi, D. Tato, C. Napoli, G. Quaranta, P. Laurenti, E. Leoni, L. G. De, C. Ossi, M. Moro, and D. G. Ribera. 2004. *Legionella* infection risk from domestic hot water. Emerg. Infect. Dis. **10**:457-464.

Bouyer, S., C. Imbert, M. H. Rodier, and Y. Hechard. 2007. Long-term survival of *Legionella* pneumophila associated with Acanthamoeba castellanii vesicles. Environ. Microbiol. **9**:1341-1344.

Brydov P., S. Uldum, N. Pringler, O.B. Jepsen. 2001. Forekomst af *Legionella* i varmtvandssystem. Identifikation og risikovurdering Miljøprojekt nr. 653 Miljøstyrelsen Miljøministeriet.

Cachafeiro, S. P., I. M. Naveira, and I. G. Garcia. 2007. Is copper-silver ionisation safe and effective in controlling *Legionella*? J. Hosp. Infect. **67**:209-216.

Castilla, J., A. Barricarte, J. Aldaz, C. M. Garcia, T. Ferrer, C. Pelaz, S. Pineda, B. Baladron, I. Martin, B. Goni, P. Aratajo, J. Chamorro, F. Lameiro, L. Torroba, I. Dorronsoro, V. Martinez-Artola, M. J. Esparza, M. A. Gastaminza, P. Fraile, and P. Aldaz. 2008. A large Legionnaires' disease outbreak in Pamplona, Spain: early detection, rapid control and no case fatality. Epidemiol. Infect. **136**:823-832.

Cramp, G. J., D. Harte, N. M. Douglas, F. Graham, M. Schousboe, and K. Sykes. 2010. An outbreak of Pontiac fever due to *Legionella* longbeachae serogroup 2 found in potting mix in a horticultural nursery in New Zealand. Epidemiol. Infect. **138**:15-20.

D´Auria G., Jiménez-Hernádez N., Peris-Bondia F. Moya A. and Latorre A. 2010. *Legionella* pneumophila pangenome reveals strain-specific virulence factors. BMC Genomics **11**.

Dorak M.T. (ed) 2006. Real-time PCR Taylor & Francis Group New York, ISBN 10:0-4153-7734-x

Edagawa, A., A. Kimura, H. Doi, H. Tanaka, K. Tomioka, K. Sakabe, C. Nakajima, and Y. Suzuki. 2008. Detection of culturable and nonculturable *Legionella* species from hot water systems of public buildings in Japan. J. Appl. Microbiol. **105**:2104-2114.

Farhat, M., M. C. Trouilhe, E. Briand, M. Moletta-Denat, E. Robine, and J. Frere. 2010. Development of a pilot-scale 1 for *Legionella* elimination in biofilm in hot water network: heat shock treatment evaluation. J. Appl. Microbiol. **108**:1073-1082.

Ferre, M. R., C. Arias, J. M. Oliva, A. Pedrol, M. Garcia, T. Pellicer, P. Roura, and A. Dominguez. 2009. A community outbreak of Legionnaires' disease associated with a cooling tower in Vic and Gurb, Catalonia (Spain) in 2005. Eur. J. Clin. Microbiol. Infect. Dis. **28**:153-159.

Flannery, B., L. B. Gelling, D. J. Vugia, J. M. Weintraub, J. J. Salerno, M. J. Conroy, V. A. Stevens, C. E. Rose, M. R. Moore, B. S. Fields, and R. E. Besser. 2006. Reducing *Legionella* colonization in water systems with monochloramine. Emerg. Infect. Dis. **12**:588-596.

Fliermans, C. B., W. B. Cherry, L. H. Orrison, S. J. Smith, D. L. Tison, and D. H. Pope. 1981. Ecological distribution of *Legionella* pneumophila. Appl. Environ. Microbiol. **41**:9-16.

Garcia, M. T., B. Baladron, V. Gil, M. L. Tarancon, A. Vilasau, A. Ibanez, C. Elola, and C. Pelaz. 2008. Persistence of chlorine-sensitive *Legionella* pneumophila in hyperchlorinated installations. J. Appl. Microbiol. **105**:837-847.

Comez-Valero L. , Rusniok C.and Buchrieser C. 2009. *Legionella* pneumophila:Population genetics, phylogeny and genomics. Infect. Genet.Evol **9**:727-739.

Guillemet, T. A., B. Levesque, D. Gauvin, N. Brousseau, J. P. Giroux, and P. Cantin. 2010. Assessment of real-time PCR for quantification of *Legionella* spp. in spa water. Lett. Appl. Microbiol. **51**:639-644.

Holden, E. P., H. H. Winkler, D. O. Wood, and E. D. Leinbach. 1984. Intracellular growth of *Legionella* pneumophila within Acanthamoeba castellanii Neff. Infect. Immun. **45**:18-24.

Hvidovre Kommune. 2001. *Legionella* i varmtvandsanlæg. Resultater fra undersøgelser I 2000.

ISO 11731-2. 2006. Dansk standard. Påvisning og bestemmelse af *Legionella* del 2: direkte membranfiltreringsmetode til vand med lavt indhold af *Legionella* 1. udgave 11.01. 2006.

Joly, P., P. A. Falconnet, J. Andre, N. Weill, M. Reyrolle, F. Vandenesch, M. Maurin, J. Etienne, and S. Jarraud. 2006. Quantitative real-time *Legionella* PCR for environmental water samples: data interpretation. Appl. Environ. Microbiol. **72**:2801-2808.

Joseph, C.A., J. Lee, J.V. Wijngaarden, V Drasar and M. C. Pastoris. 2005. European Guidelines for Control and prevention of Travel Associated Legionnaires´Disease EWGLINET.

Joseph, C. A. and K. D. Ricketts. 2010. Legionnaires disease in Europe 2007-2008. Euro. Surveill **15**:19493.

Kilvington, S. and J. Price. 1990. Survival of *Legionella* pneumophila within cysts of Acanthamoeba polyphaga following chlorine exposure. J. Appl. Bacteriol. **68**:519-525.

Kim, B. R., J. E. Anderson, S. A. Mueller, W. A. Gaines, and A. M. Kendall. 2002. Literature review--efficacy of various disinfectants against *Legionella* in water systems. Water Research **36**:4433-4444.

Krojgaard, L. H., K. A. Krogfelt, H. J. Albrechtsen, and S. A. Uldum. 2011. Cluster of Legionnaires disease in a newly built block of flats, Denmark, December 2. Euro. Surveill **16**.

Kubista, M., J. M. Andrade, M. Bengtsson, A. Forootan, J. Jonak, K. Lind, R. Sindelka, R. Sjoback, B. Sjogreen, L. Strombom, A. Stahlberg, and N. Zoric. 2006. The real-time polymerase chain reaction. Mol. Aspects Med. **27**:95-125.

LeChevallier, M. W., W. Schulz, and R. G. Lee. 1991. Bacterial nutrients in drinking water. Appl. Environ. Microbiol. **57**:857-862.

Lee, J. V., S. Lai, M. Exner, J. Lenz, V. Gaia, S. Casati, P. Hartemann, C. Luck, B. Pangon, M. L. Ricci, M. Scaturro, S. Fontana, M. Sabria, I. Sanchez, S. Assaf, and S. Surman-Lee. 2011. An international trial of quantitative PCR for monitoring *Legionella* in artificial water systems. J. Appl. Microbiol. Jan 29. doi: 10.1111/j.1365-2672.**2011**.04957.x

Leoni, E., L. G. De, P. P. Legnani, R. Sacchetti, S. Stampi, and F. Zanetti. 2005. *Legionella* waterline colonization: detection of *Legionella* species in domestic, hotel and hospital hot water systems. J. Appl. Microbiol. **98**:373-379.

Liu, Z., J. E. Stout, M. Boldin, J. Rugh, W. F. Diven, and V. L. Yu. 1998. Intermittent use of copper-silver ionization for *Legionella* control in water distribution systems: a potential option in buildings housing individuals at low risk of infection. Clin. Infect. Dis. **26**:138-140.

Mackay, I. M., K. E. Arden, and A. Nitsche. 2002. Real-time PCR in virology. Nucleic Acids Res. **30**:1292-1305.

Marciano-Cabral, F. and G. Cabral. 2003. Acanthamoeba spp. as agents of disease in humans. Clin. Microbiol. Rev. **16**:273-307.

Marrie T., P. Green, S. Burbrigde, G. Bezanson, S. Neale, P.S. Hoffman, D. Haldane 1994. *Legionella*cea in portable water of Nova Scotia hospitals and Halifax residences. Epidemiol.Infect. **112**:143-150.

Martinelli, F., A. Caruso, L. Moschini, A. Turano, C. Scarcella, and F. Speziani. 2000. A comparison of *Legionella* pneumophila occurrence in hot water tanks and instantaneous devices in domestic, nosocomial, and community environments. Curr. Microbiol. **41**:374-376.

Mathys, W., J. Stanke, M. Harmuth, and E. Junge-Mathys. 2008. Occurrence of *Legionella* in hot water systems of single-family residences in suburbs of two German cities with special reference to solar and district heating. Int. J. Hyg. Environ. Health **211**:179-185.

Moore, M. R., M. Pryor, B. Fields, C. Lucas, M. Phelan, and R. E. Besser. 2006. Introduction of monochloramine into a municipal water system: impact on colonization of buildings by *Legionella* spp. Appl. Environ. Microbiol. **72**:378-383.

Mouchtouri, V., E. Velonakis, A. Tsakalof, C. Kapoula, G. Goutziana, A. Vatopoulos, J. Kremastinou, and C. Hadjichristodoulou. 2007a. Risk factors for contamination of hotel water distribution systems by *Legionella* species. Appl. Environ. Microbiol. **73**:1489-1492.

Mouchtouri, V., E. Velonakis, and C. Hadjichristodoulou. 2007b. Thermal disinfection of hotels, hospitals, and athletic venues hot water distribution systems contaminated by *Legionella* species. Am. J. Infect. Control **35**:623-627.

Olsen D., V.F Jeppesen, L. Bagge. 2005. Undersøgelse for *Legionella* i drikkevand Miljøprojekt nr 994 Miljøministeriet.

Pringler N., P. Brydov, S.A. Uldum. 2002. Occurrence of *Legionella* in Danish Hot water systems in *Legionella* Edt: R.Marre, Y.A.Kwaik, C. Bartlett, N.P. Cianciotto, B.S. Fields, M. Frosch, J. Hacker, P.C. Lûck. ASM Press Washington DC. ISBN 1-55581-230-9.

Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil. 1994a. Influence of Plumbing Materials on Biofilm Formation and Growth of *Legionella* pneumophila in Potable Water Systems. Appl. Environ. Microbiol. **60**:1842-1851.

Rogers, J., A. B. Dowsett, P. J. Dennis, J. V. Lee, and C. W. Keevil. 1994b. Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella* pneumophila in a model potable water system containing complex microbial flora. Appl. Environ. Microbiol. **60**:1585-1592.

Rosa F. 1993. Legionnaires´disease: prevention and control Business news Publishing Company, Troy Michigan .

Saby, S., A. Vidal, and H. Suty. 2005. Resistance of *Legionella* to disinfection in hot water distribution systems. Water Sci. Technol. **52**:15-28.

Shadrach, W. S., K. Rydzewski, U. Laube, G. Holland, M. Ozel, A. F. Kiderlen, and A. Flieger. 2005. Balamuthia mandrillaris, free-living ameba and opportunistic agent of encephalitis, is a potential host for *Legionella* pneumophila bacteria. Appl. Environ. Microbiol. **71**:2244-2249.

Srinivasan, A., G. Bova, T. Ross, K. Mackie, N. Paquette, W. Merz, and T. M. Perl. 2003. A 17-month evaluation of a chlorine dioxide water treatment system to control *Legionella* species in a hospital water supply. Infect. Control Hosp. Epidemiol. **24** :575-579.

States, S. J., L. F. Conley, J. M. Kuchta, B. M. Oleck, M. J. Lipovich, R. S. Wolford, R. M. Wadowsky, A. M. McNamara, J. L. Sykora, G. Keleti, and . 1987. Survival and multiplication of *Legionella* pneumophila in municipal drinking water systems. Appl. Environ. Microbiol. **53**:979-986.

Storey, M. V., J. Winiecka-Krusnell, N. J. Ashbolt, and T. A. Stenstrom. 2004. The efficacy of heat and chlorine treatment against thermotolerant Acanthamoebae and *Legionella*e. Scand. J. Infect. Dis. **36**:656-662.

Stout, J. E., V. L. Yu, and M. G. Best. 1985. Ecology of *Legionella* pneumophila within water distribution systems. Appl. Environ. Microbiol. **49**:221-228.

Stout, J. E., V. L. Yu, Y. C. Yee, S. Vaccarello, W. Diven, and T. C. Lee. 1992. *Legionella* pneumophila in residential water supplies: environmental surveillance with clinical assessment for Legionnaires' disease. Epidemiol. Infect. **109**:49-57.

Stout, J. E. and V. L. Yu. 2003. Experiences of the first 16 hospitals using copper-silver ionization for *Legionella* control: implications for the evaluation of other disinfection modalities. Infect. Control Hosp. Epidemiol. **24**:563-568.

Swanson M.S. and B.K. hammer. 2000. Legionela pneumophila pathogenesis: A fateful journey from amoebae to macrophages. Annu. Rev. Microbiol. 54: 567-613.

Temmerman, R., H. Vervaeren, B. Noseda, N. Boon, and W. Verstraete. 2006. Necrotrophic growth of *Legionella* pneumophila. Appl. Environ. Microbiol. **72**:4323-4328.

Uldum, S.A., G. St-Martin and K. Mølbak EPI-NYT 2006-2010 http://www.ssi.dk/Aktuelt/Nyhedsbreve/EPI-NYT.aspx.

van der Kooij, D., H. R. Veenendaal, and W. J. Scheffer. 2005. Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res. **39**:2789-2798.

Wadowsky, R. M. and R. B. Yee. 1985. Effect of non-*Legionella*ceae bacteria on the multiplication of *Legionella* pneumophila in potable water. Appl. Environ. Microbiol. **49** :1206-1210.

Wellinghausen, N., C. Frost, and R. Marre. 2001. Detection of *Legionella*e in hospital water samples by quantitative real-time LightCycler PCR. Appl. Environ. Microbiol. **67**:3985-3993.

World health Organization. Copper. 2006. In: guidelines for Drinking water Quality: Recommendations. 3rd edn. Geneva: World health Organization; p 335-337.

World health Organization. Silver. 2006. In: guidelines for Drinking water Quality: Recommendations. 3rd edn. Geneva: World health Organization; p 434-435.

Yanez, M. A., C. Carrasco-Serrano, V. M. Barbera, and V. Catalan. 2005. Quantitative detection of *Legionella* pneumophila in water samples by immunomagnetic purification and real-time PCR amplification of the dotA gene. Appl. Environ. Microbiol. **71**:3433-3441.

Yanez, M. A., A. Nocker, E. Soria-Soria, R. Murtula, L. Martinez, and V. Catalan. 2011. Quantification of viable *Legionella* pneumophila cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Methods.

Yee, R. B. and R. M. Wadowsky. 1982. Multiplication of *Legionella* pneumophila in unsterilized tap water. Appl. Environ. Microbiol. **43**:1330-1334.

Zacheus, O. M. and P. J. Martikainen. 1994. Occurrence of *Legionella*e in hot water distribution systems of Finnish apartment buildings. Can. J. Microbiol. **40**:993-999.

Zhang, Z., C. McCann, J. E. Stout, S. Piesczynski, R. Hawks, R. Vidic, and V. L. Yu. 2007. Safety and efficacy of chlorine dioxide for *Legionella* control in a hospital water system. Infect. Control Hosp. Epidemiol. **28**:1009-1012.

Web pages :

http://water.epa.gov United States Environmental protection Agency

https://wwwretsinformation.dk Bekendtgørelse om vandkvalitet og tilsyn med vandforsyningsanlæg

http://www.dr.dk/Nyheder/Indland/2009/06/

http://www.oxoid.com

http: //www. solabia.fr Biokar diagnostics

9 Papers

I. Krøjgaard L.H., Krogfelt K. A., Albrechtsen H-J. Uldum S.A. Cluster of Legionnaires´ disease in a newly built block of flats, Denmark, December 2008-January 2009 Euro Surveill. 2011;16(1):pii=19759

II. Krøjgaard L.H., Krogfelt K. A., Albrechtsen H-J. Uldum S.A Validation of a quantitative real time PCR (qPCR) for detection of *Legionella* spp and *Legionella pneumophila* in water samples.

III. Krøjgaard L.H., Krogfelt K. A., Albrechtsen H-J. Uldum S.A Detection of *Legionella* by quantitative-polymerase Chain reaction (qPCR) for monitoring and risk assessment.

The papers are not included in this www-version but can be obtained from the library at DTU Environment. Please contact library@env.dtu.dk or
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